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THE MECHANISMS OF CELL DIVISION

BY

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THE MECHANISMS OF CELL DIVISION*

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CLEAVAGE AND DIFFERENTIATION IN MARINE EGGS

By Edwin G. Conklin
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I

I presume I have been invited to contribute to this monograph largely because I represent a link with the past—a kind of living fossil. It is now just sixty years since I began to study cleavage and differentiation in marine eggs, and, inasmuch as modern workers rarely refer to work that is more than twenty-five years old and never to that of the previous century, unless it be some notable monograph or compendium, it may be well that a voice from the past should recall some of the foundations on which modern cytology has been built.

In these past sixty years, I have witnessed several changes of fashion in biological research and publication, some radical changes in cytological and embryological techniques, and some revolts against older methods and results, which were hailed by their leaders as the beginnings of new eras. The older fashion of work and publication was to spend years on some research and then to publish results in a mature and comprehensive article or monograph. The newer fashion ran to shorts, weekly or monthly reports, with frequent additions and alterations. In the 1890's Jacques Loeb advised, "One idea, one paper." But the ratio was not often as simple as that; frequently ideas were inversely proportional to the square of publications. The older fashion was to avoid repetition, the newer to expect to be heard for much speaking and writing, and to gain reputation and position by a multitudinous, even if miniscule, bibliography. Professor Farlow of Harvard University once compared such progress with that of the squid, which moves rapidly backward, at the same time emitting large quantities of ink.

The so-called new eras in science have usually proved to be only stages in the continuous progress of knowledge. If one takes a long view of advancement in any field, it is evident that later work is always based on that which has gone before. A catastrophic theory of progress in science is as erroneous as a similar theory in geology and cosmogony. Such concepts are survivals of the old special creation idea according to which each species, each era, each advance is a new creation, unrelated to what has gone before. One of the most valuable results of the theory of evolution is the doctrine of the relatedness of all natural phenomena. Perhaps it is natural that younger generations should say, "Behold, we are the people and wisdom was born with us," as it is also natural that the older and disillusioned should say or think, "Lo, we were the people and wisdom will die with us." Both such opinions, however, are equally exaggerated.

In biology, one of the principal revolts of the past half century has been against the old type of morphology, which dealt only with dead materials —a revolt against the study of life with the life left out. Formerly, morphology was largely that. Professors of morphology and journals of mor-

phology dealt with structures, apart from functions. Such professorships, which were formerly found in many large universities, have now, in most institutions, been renamed professorships of biology, thus giving their holders, in title at least, a larger field of operation; and if, for bibliographical reasons, journals of morphology have retained their old names, they have generally enlarged their field by dealing with many functions as well as structures. Jacques Loeb, who denounced dead morphology and introduced the term "physiological morphology," once said to me, "I thank God I never cut a section."

But this protest against narrow morphology is applicable to many other subdivisions of biology as well as cytology, subdivisions that try to find the secrets of life in knowing more and more of less and less. William Morton Wheeler wrote contempuously of cytologists who spend their time studying the "twinkling of the centrosomes and the twiddling of the chromosomes" and neglect real life phenomena. C. M. Child maintained that "chromosomes are the expression rather than the cause of cell activity." J. Playfair McMurrich quoted with approval the saying of the botanist Sachs, "Cells do not make plants, but plants cells." Whitman's paper on "The Inadequacy of the Cell Theory of Development" emphasized a similar view and closed with this saga from the elder Huxley, "Cells, like shells on the sea-shore, mark only where the vital tides have been."

These and many other similar expressions of leading biologists emphasize the dangers of extreme specialization and the impossibility of locating all life processes in any of the parts of cells which scientific analysis has revealed. If we are ever to comprehend the nature of life we must employ synthesis as well as analysis, for life is not found in individual atoms or molecules or genes or chromosomes, but in the synthesis and organization of all of these.

The exclusive study of fixed and stained material formerly led some physiologists and experimentalists to regard chromosomes, centrosomes, spindles, *etc.* as "artifacts" caused by the treatment to which the cells had been subjected, and many biologists shared some of these views. It is curious what a vogue such opinions had, especially among experimentalists, whose methods of research invariably employ artificial modifications and isolations of normal processes. They should be cautious about denouncing similar methods on the part of cytologists. But recent work shows that the revolt against cytological "artifacts" has been greatly overdone. The most careful study of living cells shows that many of these intracellular structures are organs of the cell, organellae, and, if they are rendered more evident by fixing, staining, and sectioning, what harm is there in that? Even if some of these intracellular structures cannot be seen in living cells, even if they *are* "artifacts," at least they represent something that is different from the surrounding substance and therefore are worthy of careful study. More and more so-called "artifacts" have been shown to be normal "realfacts," however, and confidence has been restored that we have not been misled by our cytological techniques. Where would cytology be today if all that has been learned by means of such techniques were eliminated?

Several years ago, the late Dr. K. Bělăr gave a lecture at Woods Hole on certain mitotic structures and functions in the spermatogenesis of a grasshopper and, in the discussion that followed, Dr. G. H. Parker said, more or less jocosely, that he would not believe that any of these mitotic structures were real and not artifacts unless he could see them in living cells. The next morning, Bělăr invited Parker to his laboratory room to see chromosomes and spindles and asters in living cells and as he located each of these under his microscope he asked Parker if he could see them and if he believed that they were real and not artifacts. Parker looked and saw and believed, and pressing his palms together in an attitude of devotion he said, "I believe in the chromosome"; and again, "I believe in the spindle"; and finally, "I believe in the aster."

Another revolt against the old cytology, or perhaps rather a "forgetting of things which are past and pressing on to those which are before," was introduced with the application of experiments in cytology and especially with the development of cytophysics and cytochemistry. No one questions the great importance of these methods and their notable contributions to the study of cells, but why anyone should consider that these methods replace or render useless the older methods passes my comprehension.

The moral of this historical and philosophical introduction is merely this —a plea for generous recognition of the value of various kinds of cytological research and for the abandonment of the isolation of cytology from other life sciences, or worse still, of antagonism between morphologists and physiologists, observers and experimenters, for the cooperation of all kinds of biologists and indeed of all branches of science is needed in the attempt to discover the mechanisms of cell division and of the life processes that are located in cells.

II

My own studies have dealt with the cellular history of development and especially with cell division in embryonic differentiation. Just fifty-two years ago last summer, I gave a lecture at the Marine Biological Laboratory on the subject of "Protoplasmic Movement as a Factor in Differentiation," which was published in the volume of lectures for 1898, and four years later I published a monograph on nuclear and cytoplasmic movements in cell division which was entitled "Karyokinesis and Cytokinesis." These movements will be the chief theme of my remarks today. In the intervening years, I have made many experiments on the effects of pressure, electric currents, heat, cold, reduced oxygen tension, hypertonic and hypotonic solutions, various chemical reagents, centrifugal force, *etc.*, on these movements in nuclear and cell division. Much of this work has been done on the developing eggs of *Crepidula*, a marine gastropod, but the eggs of many other phyla of animals, including coelenterates, flatworms, various mollusks, annelids, ascidians, and *Amphioxus*, have been studied. But *Crepidula* has been my favorite and my figures of *Crepidula* eggs have been so numerous and perhaps so perplexing that Morgan once said he would know them in hell. Perhaps both of us may be punished in that way.

(1) Extensive studies of egg cells of many phyla have shown that many of them bear certain definite morphological relations to the mature animals that develop from them. The simplest and most general of such correlations is found in their axes. All eggs show more or less clearly a polar differentiation, in that nucleus and cytoplasm lie nearer one pole than the opposite one. The former becomes the animal or ectodermal pole, the latter the vegetal or endodermal pole of the embryo, and the axis that connects these poles marks the chief axis of the adult or lies very near to it. The relatively larger amount of yolk in the vegetal half of the egg, as compared with the animal half, retards and reduces movements in the former as compared with the latter, and the fact that yolk is heavier than cytoplasm and nucleus makes it possible to displace these in various directions by centrifugal force. Such experiments show that it is difficult or quite impossible to change permanently this chief axis of the egg. If the locations of substances at the two poles are reversed by centrifuging, they return to their original positions if given time and freedom to do so. Excellent preparations of such experiments show a cortical layer of clearer cytoplasm around the egg, which is more abundant at the animal pole than elsewhere, and, in each resting cell, strands of this cytoplasm (hyaloplasm) connect the centrosome and its surrounding centrosphere and the attached nucleus to the cortical layer nearest the animal pole. These strands of hyaloplasm may be stretched by centrifuging, but they are elastic and contractile and they pull the centrosphere and nucleus back to their original positions near the animal pole if given time and opportunity to do so. This framework of denser hyaloplasm is the structure in which the various cell inclusions are imbedded and by means of which the polarity of the egg cell persists.

In bilateral animals, the dorso-ventral and bilateral axes are established in some eggs before cleavage begins by corresponding localizations of specific cytoplasmic substances, but these localizations occur later and are less firmly fixed than in the case of the polar localizations in the chief axis. In many species, these bilateral and dorso-ventral localizations occur during or after the first cleavage, and the substances may be dislocated by mechanical or centrifugal pressure. In all cases, differential localizations become more fixed in position as development proceeds and, in some cases at least, this is associated with increased viscosity and reduced movements of the cytoplasm.

(2) When the nuclear membrane dissolves at the beginning of mitosis, the volume of karyolymph and achromatin that escapes into the cell body is much greater than the volume of euchromatin and intranuclear spindle fibers that remain in the nuclear area. Much of this escaped nuclear material gathers around the centrosome to form the asters and, in later stages, the centrospheres, as well as the astral portions of the spindle.

Some of these astral radiations reach the cortical layer of hyaloplasm and become attached to it, but before they form this attachment the spindle may be moved by centrifugal force to any position in the cell. If one pole only becomes attached, the other pole may be centrifuged to a distant position and thus the spindle fibers become stretched or bent. In such cases,

in the maturation divisions of *Crepidula*, giant polar bodies may be formed at any place on the egg, depending upon the position to which the free pole of the spindle may be centrifuged or upon the amount of stretching of the spindle, since the plane of cell division passes through the equator of the spindle.

In the maturation divisions of ascidians and other chordates which I have studied, the centrospheres and asters are poorly developed and there are no astral portions of the maturation spindles, which are barrel-shaped and purely intranuclear in origin. In other phyla, the maturation spindles, as well as all cleavage spindles, are plainly double in origin, the middle portion of the spindle being intranuclear and the polar portions extranuclear or astral. In early stages of mitosis, these two portions of the spindle may be separated by centrifuging, thus forcing the asters away from the barrel-shaped spindle with its chromosomes. Division of chromosomes takes place regularly in the barrel-shaped maturation spindles of the lower chordates and in the tissue cells of higher plants, where asters are not present; and the question arises, What function do the asters serve when they are present? There is evidence, I think, that they have to do with the location of the spindle and therewith the cleavage planes in the dividing cells—in short, with the orientations of cleavage.

With the escape of nuclear substance into the cell body at the beginning of mitosis, active movements in the nucleo-cytoplasm begin. In early stages of mitosis, the axis and position of the spindle may not coincide with its later axis and position, but these cytoplasmic movements finally bring the spindle into its proper location in the cell, unless this is forcibly prevented. If the mitotic figure forms near the center of the cell, as it does in many cases, and the resulting division should typically be unequal, cytoplasmic movements carry the spindle to an eccentric position, leaving its equator in the plane of the coming cell division, even if this should necessitate thrusting one pole of the spindle into a protrusion of the cell wall and shortening the spindle to a fraction of its previous length, in order to bring its equator into the plane of division, as is true in polar body formation in *Crepidula* and many invertebrates. In general, the axis of the spindle and its location in the cell, as well as the plane of cell division, are determined by cytoplasmic movements, but the asters, and perhaps also the hyaloplasmic framework which control these movements of orientation, are derived in part from a mixture of escaped nuclear material and cytoplasmic substances, which may be called nucleocytoplasm.

(3) The outflow of nuclear sap to the asters, through these to the cell surface, and thence back toward the equator of the cell is accompanied by a reduction of tension of the cortical layer at the poles of the spindle, elongation of the cell in the spindle axis, and by increased tension and constriction at the equator. These movements are in the form of vortices around each pole of the spindle.

In the early cleavages of *Crepidula*, where most of the cytoplasm is in the animal half of the egg and most of the yolk in the vegetal half, the vortical movements are much more extensive in the former than in the latter. Consequently, all movements toward the animal pole are much greater than

those toward the vegetal pole. As a result, the chief mass of surface hyaloplasm with the attached centrosphere and nucleus in each daughter cell is rotated toward the animal pole, while the midbody (*Zwischenkörper*), at the middle of the remains of the spindle, is carried downward in the plane of division toward the vegetal pole. Such rotations in the telophase of each cleavage continue until a late stage, but they do not carry the centrospheres under overlying cells, thus leading to a one-layered epithelium, and, in successive cleavages, the centrospheres are directed alternately to the left or right, thus causing a spiral type of cleavage.

(4) In the cycle of cell division, the chromosomes are smallest in the anaphase just after their division. Then they begin to absorb fluid from the cytoplasm and become chromosomal vesicles. As these vesicles continue to swell, they unite into a variable number of karyomeres and finally into a single nuclear vesicle in which traces of the chromosomal vesicles can still be recognized in favorable cases. The vesicular nucleus continues to take in fluid from the cell-body, apparently by endosmosis through its nuclear membrane, since visible granules never enter. This growth of the nucleus continues until the beginning of the next mitosis when the nuclear membrane dissolves, disappearing first at points opposite the centrosomes in cases where this membrane is especially thick and persistent.

The karyoplasm which escapes contains fine granules which probably could not have escaped by exosmosis, through the nuclear membrane. These granules of achromatin, oxychromatin, linin, and probably other substances, which were generated in the nucleus under the influence of the chromosomes, are liberated into the cell-body when the nuclear membrane dissolves. I have compared this gradual swelling of the nucleus and its sudden collapse to the diastole and systole of the heart, and I am convinced that it is an important feature of mitosis, perhaps as important as the division of the chromosomes. It certainly furnishes a mechanism for the formation of specific substances under the influence of the chromosomes and for starting and perhaps for directing the vortical movements in the cell body and therewith the localization of these different specific substances in the cell. These substances are then segregated in particular cells, as in cleavages in ascidians, where some of them are plainly visible and where these cells may be followed in the cell lineage until they give rise to specific organs.

In my researches on cell division and embryonic differentiation, I have paid little attention to the chromosomes and their divisions, for my material was not favorable for such work, and thereby I have "missed the bus"—perhaps I should say the band wagon! But visible differentiation takes place only in the cytoplasm, and this portion of the cell is surely worthy of more detailed study than it has generally received. After the chromosomes and genes have been more exhaustively explored, I hope that the coming generation of cytologists may turn their attention, on the one hand, to the cytoplasm and to the interrelations between this and the nucleus and, on the other, to the correlations of morphogenetic substances in the cell body with the organs of the embryo.

CELL DIVISION WITH SPECIAL REFERENCE TO CELLS IN TISSUE CULTURES*

By Warren H. Lewis
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All multicellular organisms exhibit cell division, one concern of which is the distribution of daughter chromosomes to daughter cells. Cells differ from one another as regards the chemical constitution of their different protoplasms as greatly as the adult organisms differ. If our purpose were to compare the morphology and physiology of the adults whose cells and eggs are under consideration, we would have great difficulties in finding similarities common to onions, spiderworts, seaweeds, amoebae, protozoa, snails, slugs, starfishes, sea urchins, insects, worms, fish, salamanders, rats, mice, monkeys, and men. Comparative cytology and comparative mitosis, however, reveal that all eggs and cells have a similar organization and a similar series of mitotic phases and events. They all have chromosomes, cytoplasm, and a centrosome (centroplasm). Viscosity changes of the protoplasms constituting these organs are responsible for all visible mitotic movements. When protoplasm is in the gel state, it exerts contractile tension. This is the force that produces the movements of mitosis.

Mitotic cell division involves the interaction of three series of events and movements that concern chromosomes, cytoplasm, and centrosome. Each of these self-perpetuating organs consists of its own peculiar kind or kinds of protoplasm, with its own specific metabolism and behavior. Although self-perpetuating, they are not self sufficient in environments in which cells and eggs ordinarily live. Each depends upon the others for certain substances needed for its metabolism, in addition to substances furnished by outside cell environment.

There is no direct transformation of chromosome protoplasm into cytoplasm or centroplasm, nor of the latter into each other or into chromosomes. Their metabolic products are utilized, but that is very different from the direct transformation of one sort of protoplasm into another without a preliminary digestion or breakdown of some sort. The centrosome is not a differentiated part of the cytoplasm. The so-called "*de novo*-cytasters" probably arise from detached centroplasmic astral rays.

The comments here presented are based on motion pictures of 300 dividing normal and malignant mouse and rat fibroblasts in tissue cultures, on many experiments and observations and thousands of photographs of interphase cells, on motion pictures of several early zebra fish eggs and many experiments on such eggs, on a motion picture of an early monkey egg, and on many papers by many authors too numerous to be cited here. The examination of motion pictures of cell division leads one to consider the process as a whole, beginning with an interphase cell and ending with

* Aided by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council. Motion pictures of fibroblasts and monkey egg and of zebra fish egg, shown at the time this paper was presented, were made at the Department of Embryology, Carnegie Institution of Washington, and at The Wistar Institute, respectively.

an interphase or resting daughter cell, to note all visible events, to time their onset and duration, to explain the mechanisms involved, and to distinguish between events peculiar to fibroblasts and those common to all cells.

I propose to begin with an interphase fibroblast and attempt to explain how changes of viscosity of chromosomes, cytoplasm, and centrosome bring about mitotic cell division.

Interphase Fibroblast Organization

Interphase fibroblasts consist of relatively voluminous cytoplasm, a small centrally located centrosome, and a more or less oval nucleus in a slightly eccentric position.

Sizes of Cell Organs. The factors responsible for volumes or sizes of these organs are presumably related to the sort or sorts of protoplasm which constitute them, to their peculiar metabolism requirements, to their structure or organization, and to their environment. Their volumes are probably regulated in somewhat the same manner as are liver, thyroid, parathyroid, and other organs of the adult, namely, by their metabolic interactions. Volumes may increase and decrease somewhat with changes of environment. In spite of all the work on nuclear/cytoplasmic ratios, little is known regarding the factors responsible for sizes of nucleus and cytoplasm or for the size of the centrosome.

Location of Cell Organs. The factors responsible for the central location of nucleus and centrosome have received almost no attention. Fibroblast endoplasm is usually in the gel state. It is probably more viscous at the periphery, where it grades into gel layer, than centrally. The contractile tension which it exerts thus decreases gradually from periphery to center. Owing to this gradation, all bodies, including the nucleus, are forced centerward. Greater pressure is exerted on the peripheral aspect of an embedded globule than on its deep aspect. Inclusion bodies and nucleus are pushed centerward and kept there as long as such a gelation gradient persists.

When fibroblasts cease to migrate and spread out on the cover-glass and accumulate inclusion bodies (fat globules and neutral red-staining granules), the architectural organization is often striking. Mitochondria and other inclusion bodies often become arranged radially about the centrally located centrosome in long rows except where the nucleus interferes. This radial arrangement is due to two factors, the viscosity gradient of the endoplasm, which forces nucleus and inclusion bodies toward the centrosome, and the radial centrosome rays (astral rays), which penetrate endoplasm and extend for varying distances toward the periphery of the cell into firmer endoplasm.

In active cells, however, cytoplasm is constantly undergoing local changes of viscosity. There are often temporary local solations with Brownian movement of granules. These variations tend to keep some of the mitochondria and other inclusion bodies more or less scattered in the cytoplasm, especially when cells are elongated, changing shape, and migrating.

The interphase nucleus is free. It is not attached to either cytoplasm or

centrosome. It is often moved about and even rotated by uneven pressures of the endoplasmic gel as cells change form and migrate. It tends, as already noted, to remain centrally located because of the viscosity gradient of the endoplasm. It may, however, be pushed far toward or even to the periphery, like any other large inclusion body, when many granules and vacuoles accumulate about the centrosome.

The centrosome anchors itself by protruding astral rays into the endoplasmic gel, according to its metabolic requirements. It continues to occupy the center of the cell even when the nucleus is pushed to one side by the accumulation of inclusion bodies.

Cytoplasm. The cytoplasm has a superficial gel layer and less viscous endoplasm which is usually in the gel state. They are different states of the cytoplasm which readily change from one to the other. The gel layer is the most superficial part of the cell. It is the plasma membrane. Its free superficial aspect is denser and more viscous than its deep aspect, which grades into less viscous endoplasmic gel. All cells and eggs have gel layers. Gel layers exert continuous contractile tension. Local variations of its thickness and/or viscosity produce local variations of contractile tension which are responsible for changes of cell form, protrusion and retraction of pseudopodia, flow of endoplasm, cleavage, and cell locomotion, when combined with adhesion to a substratum. When coordinated with other adherent cells, viscosity changes produce such early embryonic processes as gastrulation, invagination of lens and otic vesicle, and neural plate folding.

When cells adhere to a substratum or to one another, the surface of the gel layer becomes altered and sticky and less viscous. Adhesiveness is a surface-tension force that causes cells to spread on one another or on a substratum. It opposes the contractile tension force of the gel layer, which tends to make cells spherical. The condition of the gel layer beneath adherent areas has not been adequately tested.

Chromosomes and Nucleus. The interphase nucleus probably consists of closely packed firmly adherent swollen chromosomes (chromosome vesicles or karyomeres). Some of the evidence for this is the normal occurrence of nonadherent chromosome vesicles in invertebrates and fish, the occasional occurrence of aberrant ones in the cytoplasm of malignant fibroblasts, and the amitotic fragmentation of fibroblast nuclei into two or more parts as adhesion between the vesicles diminishes in old cultures.

Chromosomes have been depicted as consisting of chromonemata embedded in a less viscous gel matrix which is enclosed in a more viscous gel sheath.

It seems probable that the nuclear membrane is an adherent mosaic of the outer gel sheaths of the vesicles and that it does not have a cytoplasmic component. The nuclear membrane is a gel layer, and usually it becomes much thicker than the interior vesicle walls, especially in malignant cells.

The shape of the nucleus is dependent partly on external pressures and partly on the contractile tension of the nuclear membrane.

Nucleoli vary from one to six and occasionally more. The nucleolar pattern (that is, the number, size, location, and shape of the nucleoli) remains

about constant in any one cell for long periods, probably during all of interphase. It is different for each cell.

Centrosome. A centrally located centrosome can frequently be detected in living fibroblasts that are spread out on the coverglass. It is usually more or less obscured by surrounding granules and mitochrondria. Under certain conditions, its outline may be sharply defined, as in some degenerating cells. Ordinarily, it probably has astral rays which consist of centroplasm. They can protrude from and retract back to the main body of the centrosome. Centriole granules are difficult to detect in living cells, but they have been seen and photographed. They probably consist of a different sort of protoplasm.

In order to explain how it is possible for centrosomes to protrude and retract astral rays and spindle fibers and to divide, I have assumed that the centrosome has a superficial centroplasmic gel layer which exerts contractile tension and less viscous inner centroplasm. Astral rays and spindle fibers are long slender pseudopodia with an outer gel layer and a core of less viscous centroplasm. The same principles are involved in their protrusion and retraction as with cytoplasmic ones. Cleavage is accomplished by the contracting constriction of a thickened equatorial band of centrosome gel layer, as in cytoplasmic cleavage.

Outline of Mitotic Events in Fibroblasts

Chromosome Behavior. Mitotic phases are based on chromosome behavior. During prophase, chromosomes shrink and shorten into firm gel rods which can be more easily manipulated than the swollen, less viscous, chromosome vesicles of interphase. Fluid expressed from the gel collects between chromosome and vesicle wall. Shrinkage is irregular, and some parts of chromosomes become visible first as granules. Prophase is a slow process and may take from 30 minutes to an hour or more.

A few minutes before the end of prophase, the chromosome vesicle walls and nuclear membrane which consists of their outer walls solate. The free chromosomes are then pulled toward and into the metaphase plate by contractions of each pair of spindle fibers. These movements are prominent in motion pictures. They often begin by sudden twitchings, which seem to result in splitting compound nucleoli into their smaller nucleolar units. Chromosomes are pulled into the median plane of the spindle presumably because each spindle fiber tends to exert about the same tension. Chromosomes do not seem to have autonomous movements.

The movements of the chromosomes during this period and the occasional rotation of the nucleus before nuclear membrane solates suggests that the kinetochores are at the surface of the nucleus in a broad equatorial band. The simultaneous solation of vesicle walls and nuclear membrane indicates that they consist of the same sort of protoplasm, that it is different from spindle fiber gel and from endoplasm, and that the factors which produce viscosity changes in vesicle walls do not produce similar changes of cytoplasm or of centroplasm and its spindle fibers.

During metaphase, individual chromosomes oscillate as though pulled a

little toward one pole and then toward the other. This can be attributed to slight increases and decreases of the contractile tension of the spindle fibers. Chromosomes are not adherent to one another at this stage, since individual ones are freely movable. They often appear to be separated from one another by a clear zone. Oscillations continue until the daughter chromosomes separate and begin to move toward the poles. Chromosome separation is a critical event. It determines the duration of metaphase. Failure or retardation of one or more separations may result in lagging chromosomes, which escape into the cytoplasm and swell into chromosome vesicles as the daughter nuclei swell. Metaphase duration for normal fibroblasts is from $3\frac{1}{2}$ to 12 minutes and for malignant ones 12 to 50 minutes.

During anaphase, chromosomes are pulled by contraction of spindle fibers to each pole, where they adhere to one another in a short truncated cone with projecting chromosome ends. The centrosome is at the apex. A homogenous fluid, exnuclear or vesicle sap, remains behind between the two groups of chromosomes. Anaphase duration is 1 to $3\frac{1}{2}$ minutes for normal fibroblasts; 2 to 6 for malignant ones.

During telophase, each daughter chromosome swells into a vesicle. They remain tightly adherent and tend to spread on one another until surface tension is reduced to a minimum. Swelling is uneven. The nucleus soon has a granular appearance similar to the prophase one. As swelling continues, all visible traces of the chromosomes disappear from view, except the granular nucleolar units. The latter agglutinate into one or more nucleoli that remain constant in number, size, shape, and location. When the nucleolar pattern becomes established, telophase may be considered as ended. Nucleolar agglutination is a haphazard process, for no two nucleolar patterns are exactly alike, and even those of daughter cells differ from each other and from the mother cell.

Telophase swelling may begin at about the time the chromosomes reach the poles or a little later. One might consider telophase as beginning with the swelling, but it is more usual to consider it as beginning when the chromosomes reach the pole. Telophase duration is difficult to determine. It probably takes one to two hours.

Cytoplasm Behavior. All visible cytoplasmic movements are due to viscosity changes. The first visible movement is retraction or partial retraction of pseudopodia and cell processes, a corresponding enlargement of the cell body and a tendency for the latter to become spherical. These events are the result of a decrease of viscosity of endoplasm and gel layer of the cell body during prophase, an event that occurs in all cells. In egg cells, decrease of endoplasmic viscosity results in a marked increase of cytoplasmic granular movements, a conspicuous phenomenon in motion pictures of rabbit, monkey, and fish eggs. Such movements are not apparent in fibroblasts.

Pseudopod retraction can be compared to the contracting posterior ends of migrating cells and amebae; the enlarging cell body, to the bulging anterior end or pseudopod. The contractile tension of the gel layer of the pseudopod forces its less viscous endoplasmic core into the cell body as gel

layer of the latter relaxes. This continues as long as the contractile tension of the pseudopod gel layer is greater than that of the gel layer of the cell body. As the pseudopod gel layer contracts, its inner aspect solates into endoplasm, which is also squeezed into the cell body.

The next visible cytoplasmic event is the equatorial flattening of the cell during anaphase. It produces mitotic cell elongation. It is due to an increase of the viscosity and/or thickness of a broad equatorial band of the gel layer, which results in increased contractile tension. This is probably a part of a general increase of viscosity of endoplasm and gel layer that begins in late metaphase, continues during anaphase and early telophase, and persists until the end of cleavage. In eggs, this general increase of cytoplasmic viscosity tends to fix granular inclusions *in situ* until cleavage is ended. During mitotic cell elongation, the spindle-shaped exchromosomal sap is compressed into an elongated cylinder which forces the two groups of daughter chromosomes farther apart.

Cleavage, which follows soon, is due to still greater increases in the thickness and/or viscosity of a narrow equatorial part of the broad equatorial band of the gel layer. The increased contractile tension of the narrow constriction band constricts the cell in two. As it contracts, it bends endoplasm inward until it divides the softer exchromosomal or spindle sap. The constriction band then divides endoplasm and constricts to a stalk which connects the two daughter cells for a short time. After division, the exchromosomal sap mixes with endoplasm. The bending inward of the endoplasm (which often contains a row of fat globules) indicates that it is in the gel state and more viscous than the exchromosomal sap. The constriction cleft usually begins to show at about or after the end of anaphase, rarely before. The duration of cleavage is from one to seven minutes for normal fibroblasts and two to thirteen minutes for malignant ones.

Pseudopod protrusion usually begins immediately after cleavage, and at or near the region where the old pseudopod was retracted. New pseudopods usually follow the paths of old ones as they move out on the surface of the coverglass. Pseudopod protrusion is probably due to a local decrease of viscosity and/or thickness of the gel layer at the poles.

Bleb formation is a conspicuous event. It varies greatly in time of origin, duration, and location. It is occasionally absent. It rarely occurs on egg cells. It usually begins at the poles or on the processes but may begin on the cell body or near the equator. Blebs are small pseudopods that protrude and retract at small weak areas of the gel layer.

Centrosome Behavior. Centrosome migration and spindle fiber formation have not been followed in living fibroblasts. The movie of the monkey egg shows a lateral view of one of the blastomeres. It has a granular centrosome mass adjacent to the nucleus. The centrosome itself is obscured by peculiar dark granules. This mass increases in size and divides into two unequal parts. The smaller one moves over the surface of the nucleus to the opposite pole before any nuclear changes are apparent. Each centrosome then divides and immediately fuses into a single mass. At this time, the nucleus disappears from view. It probably goes into prophase. A

polar view of another blastomere shows a small clear centrosome surrounded by a thick zone of granules. Centrosome migration and division would probably not be detected without the accompanying granules. Chromosomes, astral rays, and spindle fibers, although present, have not been seen.

The idea that the centrosome has a gel layer which is subject to local variations of contractile tension offers one step in an attempt to explain the mechanism of centrosome cleavage, astral ray, and spindle fiber formation. Cleavage may be accomplished by the contracting constriction of an equatorial band of the gel layer, which has increased in thickness and/or viscosity and hence in contractile tension. It is accomplished in the same manner as cytoplasmic cleavage. The factors responsible for the production of the equatorial band are probably related to changes that occur in the centrosome.

Astral rays and spindle fibers are centrosome pseudopodia that are protruded and retracted in much the same manner as are cytoplasmic ones. Astral rays probably continue to protrude at the same time that spindle fibers are retracting or exerting contractile tension, as during metaphase or anaphase. It is not uncommon to see some cytoplasmic pseudopodia protruding while others, not far distant on the same cell, are retracting. The extreme slenderness of astral ray and spindle fiber pseudopodia is to be explained by peculiarities of centrosome protoplasm, just as long slender nerve axons are due to certain peculiarities of nerve cell protoplasm, or the blunt pseudopodia of amebae and slime molds or the membraneous ones of macrophages are due to their peculiar cytoplasms.

Summary

Fibroblast mitosis depends on the interaction of three interdependent self-perpetuating cell organs—chromosomes, cytoplasm, and centrosome.

Interphase Cell. The nucleus consists of firmly adherent swollen chromosomes. Their outer walls form a mosaic nuclear membrane with kinetochores at the surface. The cytoplasm consists of a superficial gel layer and less viscous gelated endoplasm which exert contractile tension. The centrosome probably has its own gel layer which also exerts contractile tension. The central location of the centrosome, nucleus, and inclusion bodies is due to the contractile gradient of the endoplasm.

Mitotic Events. The duplex chromosomes shrink and become free during prophase, split into daughter chromosomes at the end of metaphase, adhere and swell to form the interphase nucleus during telophase. They are moved by spindle fibers during prophase-II, metaphase, and anaphase.

Cytoplasmic pseudopods retract during prophase and metaphase as contractility and viscosity of the cytoplasm of the cell body decreases. Increased contractility of the equatorial region of the cytoplasm causes cell flattening and elongation during anaphase and cleavage by constriction during early telophase. During cell flattening and cleavage, the spindle sap (exchromosomal sap) is elongated. This pushes centrosomes and attached chromosomes toward cell poles.

Hypothetical Outline of Centrosome Behavior. During early prophase, the

centrosome divides, one part migrates to opposite pole of the nucleus, each then divides and immediately fuses into a duplex body. Each one then protrudes pseudopods (spindle fibers) to the kinetochores. After attachment, pseudopods become retractile and pull freed chromosomes into the median plane of the spindle and then the daughter chromosomes to the poles. Each pseudopod has a contractile gel layer and a less viscous centroplasmic core.

Conclusion. Viscosity changes and contractile tensions exerted by protoplasm in the gel state are responsible for all visible mitotic movements.

CYTOPLASMIC CHANGES DURING CELL DIVISION WITH REFERENCE TO MITOCHONDRIA AND THE GOLGI SUBSTANCE

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After the discovery of the mitochondria by Flemming[1] and Altmann[2] and the long series of papers demonstrating that the mitochondria are practically omnipresent in living cells,[3] many investigations were made in an effort to determine their functional significance. Nothing definite may be concluded from this early work except that mitochondria are possibly concerned in some way with respiratory activity of the cytoplasm.[4]

Similarly, since the discovery of the Golgi substance by Golgi in 1898,[5] numerous attempts have been made to determine its significance, the most successful being those of Bowen,[6] dealing with spermatogenesis. While his work indicated that the Golgi substance is concerned with the formation of the acrosome during maturation of the sperm, the significance of this cytoplasmic component in mature cells of other types has not been clearly defined. The most generally accepted hypothesis is that it serves as an area for the segregation and accumulation of the products of secretory or excretory activity of the cell.[7–11]

Included in this early work were a few studies on the mitochondria and Golgi substance during cell division. Cowdry[12] found no significant change in mitochondrial number during cell division in embryonic cells, and Ludford[13] demonstrated fragmentation and dispersion but not disappearance of the Golgi substance during mitosis in tumor cells.

Recent studies on the detailed morphology of mitochondria[14–17] and the Golgi substance [11, 18] and on the chemical characterization of mitochondria[19] give further evidence that both of these cellular components play an important role in cellular metabolism. The presence of a mitochondrial membrane,[14–17] the extreme sensitivity of mitochondria to changes in osmotic pressure,[17] and the fact that they are apparently the sole carriers of certain enzymes[19–20] are all suggestive leads.

It was with this background in mind that it was decided to reinvestigate the possible changes in mitochondria and the Golgi substance during cell division. With few exceptions, normal adult tissue is not the material of choice for studies on cell division because of the relative scarcity of mitotic figures. It was decided to investigate a series of spontaneous and induced transplantable tumors in mice collected over a period of six years at the National Cancer Institute. This series included a group of sarcomas: CR-180, S-37, a spontaneous fibrosarcoma,[21] an osteogenic sarcoma,[22] and the H, J, and *O* series of Earle sarcomas;[23] and a group of lymphomas including one thymoma and a series of epithelial tumors: squamous cell carcinoma, hemangio-endothelioma,[24] spontaneous adrenal cortical tumors,[25] and spontaneous and induced hepatomas.[18] While other tumor types showed similar

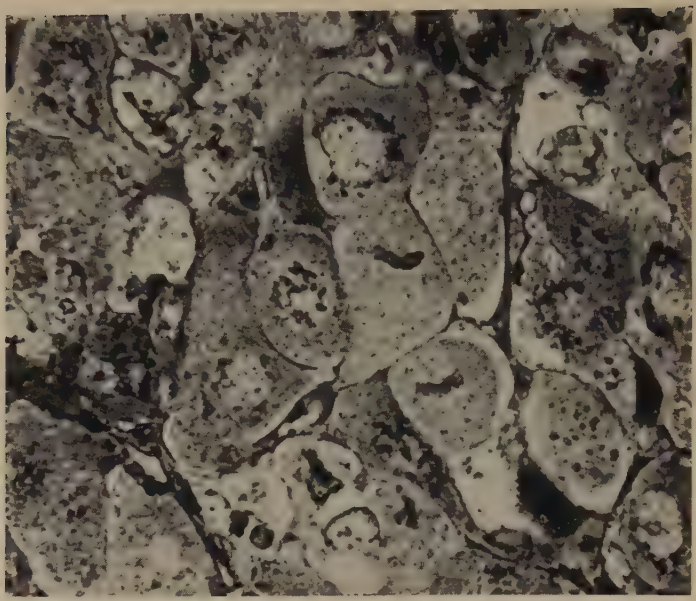

FIGURE 1. Two metaphase and two prophase figures from transplanted C Cl_4 induced hepatoma showing absence of osmiophilic material. Kolatchev-Nassonov method × 760.

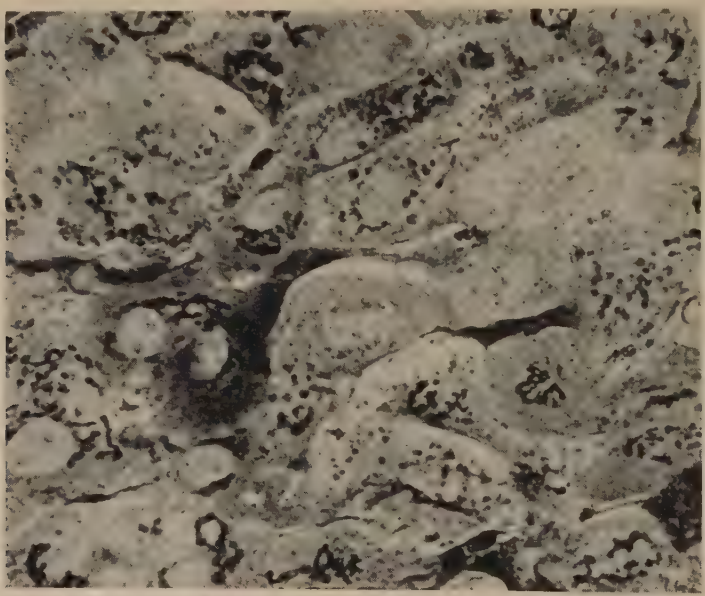

FIGURE 2. A cell in anaphase from a transplanted C Cl_4 induced hepatoma showing absence of osmiophilic material. Kolatchev-Nassonov method × 760.

changes, the tumors chosen for intensive study were the spontaneous adrenal cortical tumors and the induced and spontaneous hepatomas, because of the

large cell size and large mitochondrial size. In addition, the Golgi substance is present in large amounts in the resting cells of both spontaneous and induced hepatomas.

The Regaud method was used for visualizing the mitochondria, and the Kolatchev-Nassonov technique was used to impregnate the Golgi substance. With but rare exceptions, all hepatoma cells in division contained no visibly demonstrable Golgi substance. In areas where all cells in the resting stage contained large amounts of heavily impregnated Golgi substance, cells in mitosis could be identified readily by the complete lack of osmiophilic material in their cytoplasm. FIGURE 1 shows a group of two prophase and two metaphase figures surrounded by cells in the interkinetic state. FIGURE 2 shows a cell in anaphase surrounded by resting cells. The absence of osmiophilic material in the cells in division in FIGURES 1 and 2 is obvious.

Tumor cells may be separated into two groups on the basis of the reaction of their contained mitochondria to cell division. The larger group, into which the majority of the tumor cell types studied may be placed, is exemplified by the spontaneous adrenal tumors. In the cells of these tumors, there is no obvious change in mitochondrial number during mitosis. There is a change, however, in the staining reaction of mitochondria. FIGURE 3 shows a cell in prophase. The mitochondria in this cell appear to be equal in number and similar in their staining reaction to those in the surrounding resting cells. At metaphase however, the mitochondria appear to be somewhat smaller and are thus stained more lightly than those in resting cells (FIGURE 4). During anaphase, there was no apparent change from that seen at metaphase, but the mitochondria of cells in telophase appear to stain more heavily than those in resting cells (FIGURE 5).

The other type of mitochondrial reaction to cell division is characteristic of mitochondria of the cells of spontaneous and induced hepatomas in the mouse. In cells of these tumors, the mitochondria decrease markedly in number in prophase. FIGURE 6 shows a cell in prophase in an induced hepatoma, obviously containing fewer mitochondria than the surrounding cells.

The mitochondria also appear to be more lightly stained. FIGURE 7 shows a cell, in somewhat later prophase than that in FIGURE 6, in a spontaneous hepatoma. The decrease in number and change in staining reaction of the mitochondria are obvious. By the time metaphase is reached, the mitochondria appear to approximate in number those in surrounding resting cells (FIGURE 8), but they still appear to be more lightly stained. Compared with the mitochondria of resting cells, their diameter is less.

Discussion

The idea of the *de novo* origin of the Golgi substance is by no means a new one. Cramer and Ludford[26] implied it in their studies on the cytology of hepatic cells, suggesting that the Golgi substance originated near the nucleus and migrated to the periphery of the cells, disappearing at the bile capillary. Thus, continual synthesis and break down or loss of Golgi substance was implied. The present study, showing complete disappearance

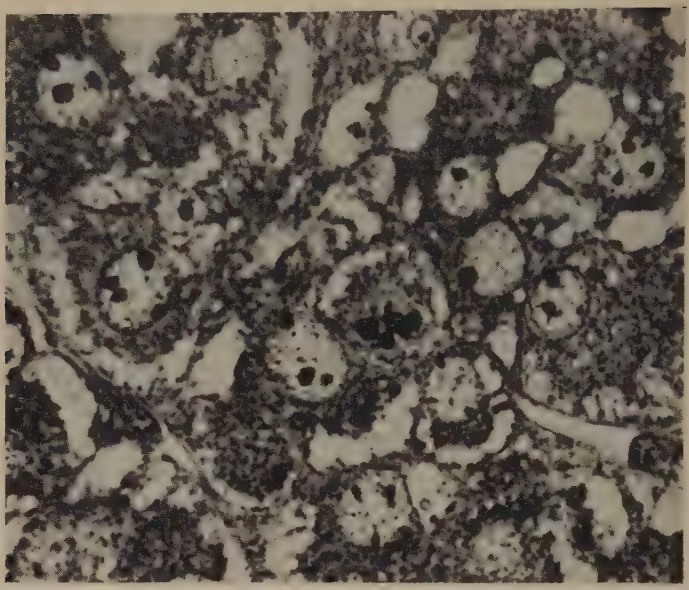

FIGURE 3. A cell in prophase from a spontaneous adrenal cortical tumor, showing the mitochondria to be approximately equal in number and similar in staining reaction to those in surrounding cells. Regaud fixation. Iron hematoxylin stain × 1040.

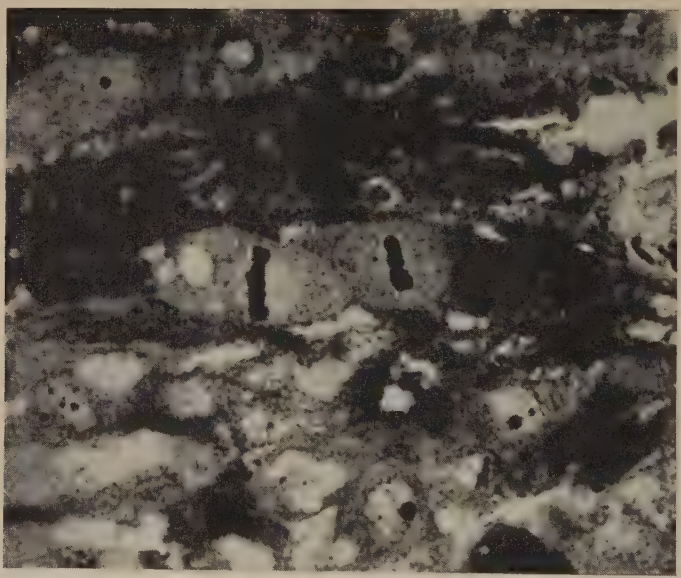

FIGURE 4. A cell in metaphase from a spontaneous adrenal cortical tumor showing lightly stained mitochondria. Regaud fixation. Iron hematoxylin stain × 1040.

of the Golgi substance during mitosis in hepatoma cells, is reasonably strong evidence in support of the *de novo* origin of the Golgi substance, since it is consistently present in large amounts in the resting cells of this tumor.

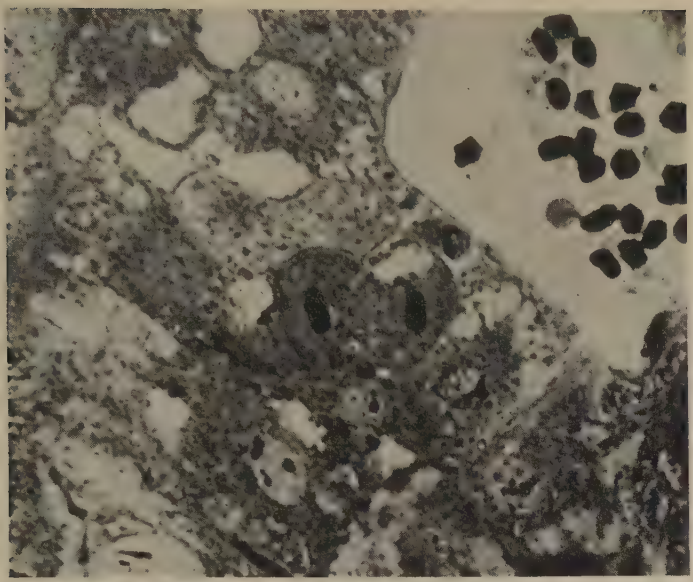

FIGURE 5. A cell in telophase from a spontaneous adrenal cortical tumor showing the mitochondria to be more deeply stained than those in surrounding cells. Regaud fixation. Iron hematoxylin stain × 1040.

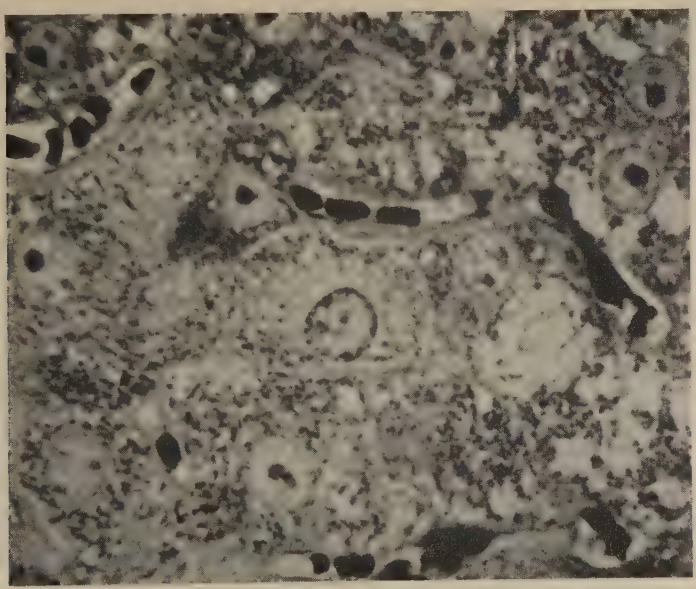

FIGURE 6. A cell in early prophase from a CCl_4 induced hepatoma showing a reduction in the number of mitochondria. Regaud fixation. Iron hematoxylin stain × 1040.

It is, of course, possible that diffusion of substances from the nucleus into the cytoplasm at the onset of mitosis could result in a change in the Golgi substance so that it no longer would react with osmic acid. This is appar-

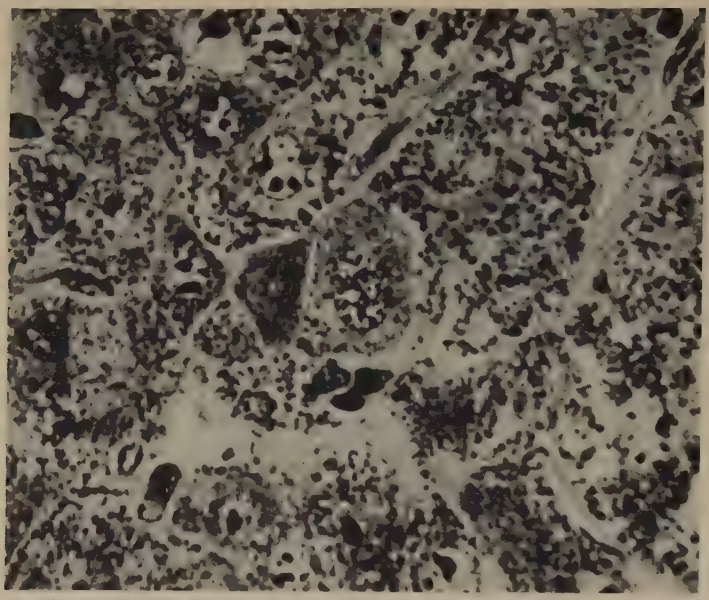

FIGURE 7. A cell in early prophase from a spontaneous hepatoma showing an obvious reduction in the number of mitochondria. Regaud fixation. Iron hematoxylin stain × 1040.

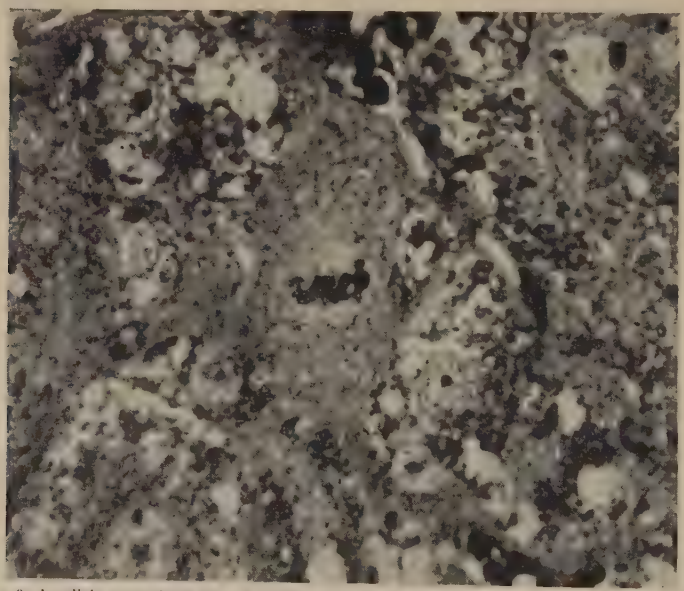

FIGURE 8. A cell in metaphase from a C Cl_4 induced hepatoma containing pale staining mitochondria approximately equal in number to those in surrounding cells. Regaud fixation. Iron hematoxylin stain, × 1040.

ently not the case in all types of tumor cells, however, since, as mentioned earlier, Ludford[13] found fragmentation and dispersion but not disappearance of the Golgi substance during mitosis in certain tumor cell types.

Of the multitude of suggestions as to the probable function of mitochondria,[3] the most reasonable is that they are concerned with the general metabolism of the cytoplasm. The demonstration of their presence in all cell types in which they have been sought, with the exception of bacteria and the blue-green algae, is in itself suggestive of their importance. The presence of a membrane[14–17] and the probability that they are the exclusive carriers of certain enzymes[19–20] suggest that they are qualitatively different from cytoplasmic protoplasm *per se*.

Earlier workers,[27–29] visualizing them as bearing factors concerned with cytoplasmic inheritance, suggested that the mitochondria divide qualitatively, as do chromosomes. While this hypothesis has not been supported by later work,[3] the evidence which is accumulating indicates more and more clearly their qualitative difference from the ground substance of the cytoplasm.

The changes reported here suggest that mitochondria are definitely affected by, and possibly involved in, the metabolic changes which occur during cell division.

Bibliography

1. Flemming, W. 1884. Mittheiluug zur Farbetechnik. Z. f. wiss. Mikr. **1:** 349–361.
2. Altmann, R. 1894. Die Elementaroganismen und ihre Beziehungen zu den Zellen. Leipzig.
3. Guilliermond, A. 1934. Les constituants morphologiques du cytoplasme: Le chondriome. Actualites Scientifiques et Industrielles. Exposes de Biologie **170:** 1–128.
4. Cowdry, E. V. 1924. General Cytology, Section 6. University of Chicago Press. Chicago.
5. Golgi, C. 1898. Interno alla struttura della cellule nervose. Bull. Soc. Med. Chir. di Pavia.
6. Bowen, R. H. 1929. Cytology of glandular secretion. Quart. Rev. Biol. **4:** 484.
7. Kirkman, H. & A. E. Severinghaus. 1938. A review of the Golgi apparatus. Anat. Rec. **70:** 413–429, 557–573.
8. Hirsch, G. C. 1939. Protoplasma-Monographien **18:** Form.-und Stoff weschel der Golgi-Körper. Verlog von Gebruder Borntraeger. Berlin.
9. Hibbard, H. 1945. Current status of our knowledge of Golgi apparatus in the animal cell. Quart. Rev. Biol. **20:** 1–19.
10. Worley, L. G. 1946. The Golgi apparatus—an interpretation of its structure and significance. Ann. N. Y. Acad. Sci. **47:** 1–56.
11. Bourne, G. 1942. Cytology and Cell Physiology (4): 99–138. The Clarendon Press. Oxford.
12. Cowdry, E. V. 1914. The relations of mitochondria in cells multiplying by mitotic and amitotic division. Anat. Rec. **8:** 102.
13. Ludford, R. J. 1924–25. The distribution of the cytoplasmic organs in transplantable tumour cells, with special reference to dictyokinesis. Proc. Roy. Soc. Lond. **97:** 50–60.
14. Claude, A. & E. F. Fullam. 1945. Electron microscope study of isolated mitochondria; method and preliminary results. J. Exper. Med. **81:** 51–62.
15. Dalton, A. J., M. G. Kelly, H. B. Kahler, & B. J. Lloyd. 1947. Some observations on the isolation of mitochondria of hepatic cells of the mouse by differential centrifugation. Presented at International Cancer Research Congress, St. Louis, Mo. Sept.
16. Dalton, A. J., H. B. Kahler, B. J. Lloyd, M. J. Striebich, & P. B. Maury. 1948. Further observations on the mitochondria of normal hepatic cells and of hepatoma. Presented at Meeting of American Association of Anatomists, Madison, Wis. April.
17. Zollinger, H. U. 1948. Cytologic studies with the phase microscope. II. The mitochondria and other cytoplasmic constituents under various experimental conditions. Am. J. Path. **24** (3): 569–590.
18. Dalton, A. J. & J. E. Edwards. 1942. Mitochondria and Golgi apparatus of induced and spontaneous hepatomas in the mouse. J.N.C.I. **2** (6): 565–575.

19. Hogeboom, G. H., W. C. Schneider, & G. E. Pallade. 1948. Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. J. Biol. Chem. **172:** 619–636.
20. Kennedy, E. P. & A. L. Lehninger. 1948. Intracellular structures and the fatty acid oxidase system of rat liver. J. Biol. Chem. **172:** 847–48.
21. Edwards, J. E., A. J. Dalton, J. White, & T. N. White. 1942. A spontaneous fibrosarcoma of the foreleg and paw in a C3H mouse. J.N.C.I. **3** (2): 191–198.
22. Barrett, M. K., A. J. Dalton, J. E. Edwards, & J. P. Greenstein. 1944. A transplantable osteogenic sarcoma originating in a C3H mouse. J.N.C.I. **4** (4): 389–402.
23. Earle, W. R. & A. Nettleship. 1943. Production of malignancy *in vitro. v.* results of injections of cultures into mice. J.N.C.I. **4:** (2): 213–227.
24. Edwards, J. E., H. B. Andervont, & A. J. Dalton. 1942. A transplantable malignant hemangio-endothelioma of the liver in the mouse. J.N.C.I. **2** (5): 479–490.
25. Dalton, A. J., J. E. Edwards, & H. B. Andervont. 1943. A spontaneous, transplantable, adrenal cortical tumor arising in a strain C mouse. J.N.C.I. **4** (3): 329–338.
26. Cramer, W. & R. J. Ludford. 1926. On the cellular mechanism of bile secretion and its relation to the Golgi apparatus of the liver cell. J. Physiol. **62:** 74–80.
27. Meves, F. 1918. Die Plastosomentheorie der Vererbung. Arch. f. mikr. Anat. **92** (2): 41–136.
28. Meves, F. 1918. Eine neue Stutze fur die Plastosomentheorie der Vererbung. Anat. Anz. **50:** 551–57.
29. Duesberg, J. 1919. On the present status of the chondriosome problem. Biol. Bull. **36:** 71–81.

CONTROL OF FISSION IN *AMOEBA PROTEUS* AS RELATED TO THE MECHANISM OF CELL DIVISION

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The experimental control of the process of fission in *Amoeba* has shown that the mechanism is of some complexity, as might be expected from the kaleidoscopic changes in morphology that are seen.

The results taken *in toto*, however, seem to permit the division of the process into two parts: first, the division of the nucleus and, second, the division of the cell body. It also seems that the mechanisms active in these two stages, while acting integratively to complete fission, are to a considerable degree separable.

This concept is based on experiments carried on over a period of some fourteen years, which experiments had for their object the elucidation of the mechanisms of cell division in *Amoeba*. The results obtained have not been brought together as a whole. This monograph offers the author an opportunity to make such a survey. It is his hope that the views expressed will not prove anachronistic in the extreme.

In presenting the concepts for discussion, a reversal in the time sequence of the events of fission is made, for the reason that, experimentally, control is most effectively achieved in that order. Therefore, discussion of division of the cell body, and its control, will precede discussion of division of the nucleus and its control. But first, it would be well to give a brief general description of the dividing cell.

Amoeba preparing for fission becomes nearly spherical and presents a surface studded with fine pseudopodia. This form change is coincident with the entry of the nucleus into prophase. For about twenty-five minutes, the cell remains quiet, except for a continuous withdrawal and replacement of pseudopodia, which gradually increase in diameter. Then, quite suddenly, the cell elongates, pseudopodia of relatively large size develop at the poles and attach to the substrate, and, viewed grossly, normal locomotion is resumed in opposite directions at the two poles and gradually draws the mother cell apart to form the daughter cells. Microscopically, the picture is more complex. After a slight extension of the cell, a rather vague furrow appears at the cell equator but develops somewhat incompletely. The flow of cytoplasm is not directed from the furrow toward the poles but can be seen coursing alternately toward one or the other of the daughter cells through the narrowing waist between them. The direction of flow is correlated in time and extent with the pseudopodial formation at the poles, being directed always toward the most active region. This process continues until the increasing locomotor activity literally tears the daughter cells apart. When the last cytoplasmic thread snaps, the severed ends fly apart in a way that shows it to be a highly elastic filament that fails under tension.

During the process of cytoplasmic fission, the nuclear division is completed. The beginning of elongation with extension of the polar pseudopodia is coincident with the approach of the daughter nuclei to the cell surface during late anaphase.

Measurement of the contractile vacuole or vacuoles during fission indicates that the expulsion of fluid from the cell decreases during fission and, at metaphase, is briefly replaced by intake from the vacuole into the cytoplasm.[1]

The impression is forcible that locomotion plays a leading role in cytoplasmic fission, and, probably, that great changes take place in the intracellular colloidal conditions, fluid intake replacing fluid expulsion during a portion of the process.

Control of Cytoplasmic Fission. It is of interest to examine the type of reagent effective upon the velocity, *etc.*, of cytoplasmic fission. There are many reagents which will inhibit or produce abnormalities of fission of both nucleus and cytoplasm. In general, these are heavy metals, some narcotics, or general protoplasmic poisons, and their action is of a toxic nature, affecting the entire cell. Of greater interest, since they indicate the separation of the mechanisms, are those which affect cytoplasmic fission but have little or no effect on karyokenesis.

Acids, (particularly certain organic acids such as pyruvic and lactic acids), bases, salts of the light metals, and combinations thereof have been shown[2] to influence this process. All these agents modify the locomotor activity of the daughter cells, and certain things (lactic acid in particular) noticeably affect the colloidal state of the cytoplasm, as evidenced by marked changes in the sol-gel ratio. Any reagent, including mechanical interference with a fine glass needle, which prevents adhesion to the substrate, acts to inhibit division of the cytoplasm. These reagents are well known for their effects on ameboid locomotion, but, in their presence, nuclear fission in *Amoeba* proceeds normally, as evidenced by the normality of the time sequence of its phases. One exception was found, and that only a partial exception to the rule, that any interference with locomotion, and particularly any interference with adhesion of the cell to the substrate, prevented fission of the cell. This exception was exposure to glass-distilled water. *Amoeba* cannot adhere to the substrate when in distilled water. Therefore, while pseudopodia formation proceeds as usual, traction cannot be exerted to divide the cytoplasm. Experiments showed, however, that sixty to sixty-five per cent of cells so exposed completed fission.[1]

Examination of the fission process under these conditons furnished the explanation. FIGURE 1 shows diagrammatically what occurred. The polar pseudopodia at both poles recurved, and, if the opposing groups of pseudopodia impinged, as was usual, on their opposites, the cell, by flowing into these while continuing to contract at its equator, was able to exert tension and finally rupture the bridge between the daughter cells. If the pseudopodia slipped past each other, they continued to extend, but no tension could result. The cell became stellate, floated for some time in this state, and finally, after a time, became a binucleate. No further attempt at fission then occurred until a new mitotic cycle ensued.

It is to be noted that the entire cytokinetic process fits well with the ideas of Mast[1, 3] as to the mechanism of locomotion in *Amoeba*, and that the experimental reagents that interfere with cell, but not with nuclear, fission are among those characteristically employed to influence locomotion or attachment to the substrate. One reagent of considerable interest is alizarin sulphonate, found by Pollack[4] to inhibit entirely locomotion and even elongation. TABLE 1 shows that it was almost completely effective in preventing cytoplasmic fission.

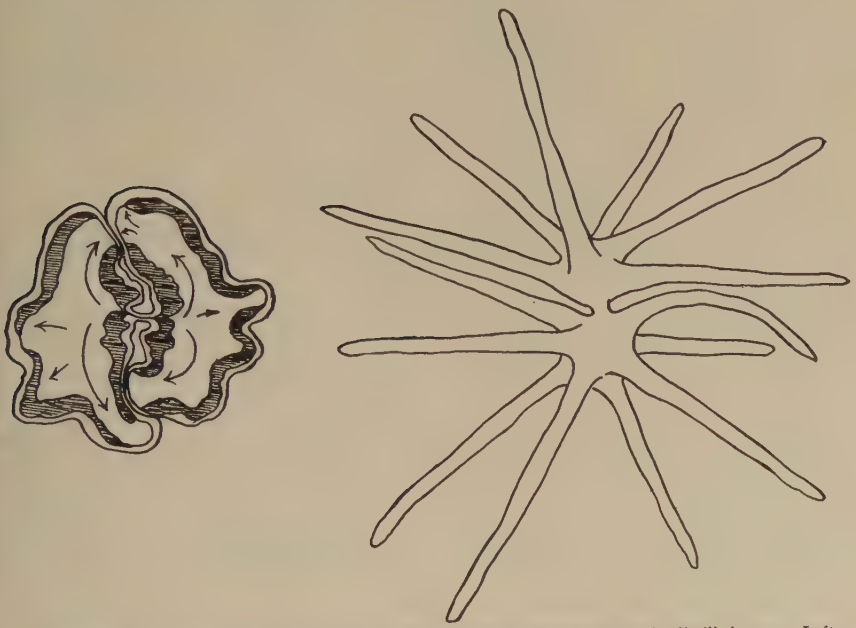

FIGURE 1. Diagrammatic sketch illustrating method of fission in *Amoeba proteus* in distilled water. Left—successful. The tips of the pseudopodia at a and b are pressing against those of the opposing cell. This pressure is continued and the pseudopodia grow, thus tending to lengthen the bridge between the cells and remove the plasma gel in the bridge. This stretching and thinning of the bridge results in eventual separation. Right—failure. The opposing pseudopodia fail to meet, or slip after meeting. No strain can be set up and the cells become stellate and remain attached.

It was noted above that all *Amoebae* that failed to complete fission in distilled water remained binucleates until a new cycle of mitosis involving one or both nuclei supervened. This suggested that the nucleus during its fission might serve as a continuing stimulus to cytokinesis, but that the stimulus to division of the cell body faded with the completion of the nuclear cycle.

A study of distribution in time of fission under normal conditions and under inhibition by a number of agents is presented in FIGURE 2. It will be seen from this figure that, while the duration of cytokinesis, for all *Amoebae* that succeeded in dividing, varied from eight to forty-seven minutes after elongation, it plainly indicated that nuclear fission had occurred and that all curves shown tend to reach minima about 26 minutes after elongation. This shows that, if an *Amoeba* has not completed cell division in this twenty-

TABLE 1

EFFECT OF ALIZARIN SULPHONATE ON CELL DIVISION IN *Amoeba proteus*
(ALL EXPOSED 15 MIN.)

M/1000 no. am.	*Time to elongation*	*Time to fission*	*Nuclear condition after 12 hrs.*
1	5 min.	16 min.	
2	did not elongate	failed	Bi
3	did not elongate	failed	Bi
4	did not elongate	failed	Bi
5	did not elongate	failed	Mono[1]
6	did not elongate	failed	

M/500 no. am.	*Time to elongation*	*Time to fission*	*Nuclear condition after 12 hrs.*
1	did not elongate	failed	Bi
2	did not elongate	failed	Bi
3	did not elongate	failed	Bi
4	did not elongate	failed	Bi
5	did not elongate	failed	Bi

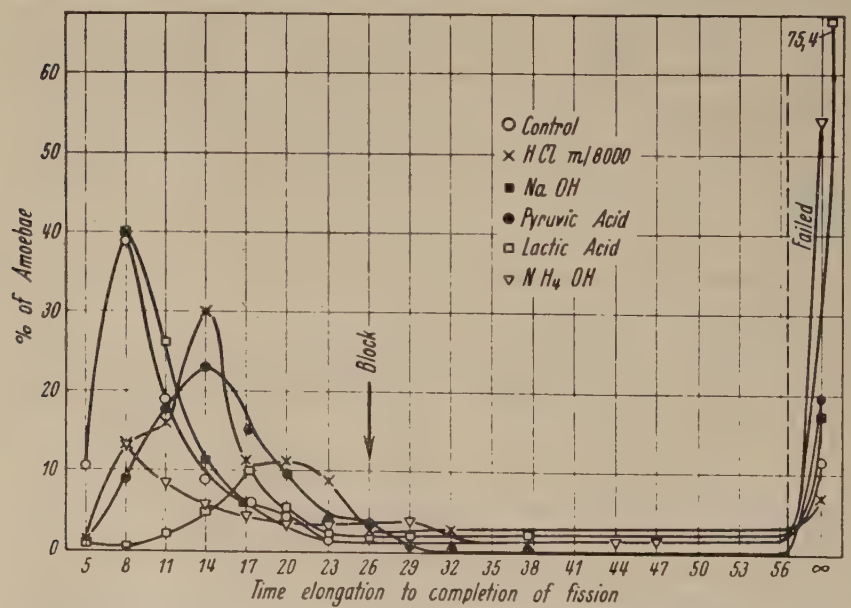

FIGURE 2. Graph showing the distribution of the times required for cytokinesis as measured from elongation to completion of fission in: saline; m/8,000 lactic acid; m/15,000; pyruvic acid m/40,000 NaOH; m/40,000 NH_4OH. Note that although the mode is shifted from about 8 minutes in lactic acid, all curves tend to a minimum at about 26 minutes, indicating a block to fission after this time.

six minute interval, it usually remains as a binucleate until a new mitotic cycle arises.

This finding caused examination to be made of the state of the daughter nuclei during this 26-minute period. Evidence was found[1] that, coincident

with the loss of tendency to complete cytokinesis, the restoration of interkinetic morphology in the nucleus occurred, as evidenced by restoration of refractive index (the nucleus became more brilliant than the rest of the cell), and restoration of the interkinetic distribution of granules beneath the nuclear membrane also occurred. It is also to be noted that the two nuclei appear to remain in close juxtaposition after this time. Before, they are always seen to be well separated.

At approximately the same time, the nucleus recovers the differential staining for sulphydryl that, as noted later, is characteristic of the resting nucleus.[5]

Summary. The process of cytoplasmic fission in *Amoeba* appears to be experimentally controllable by an agent that will affect locomotion or attachment to the substrate. It is therefore suggested that this fact, taken

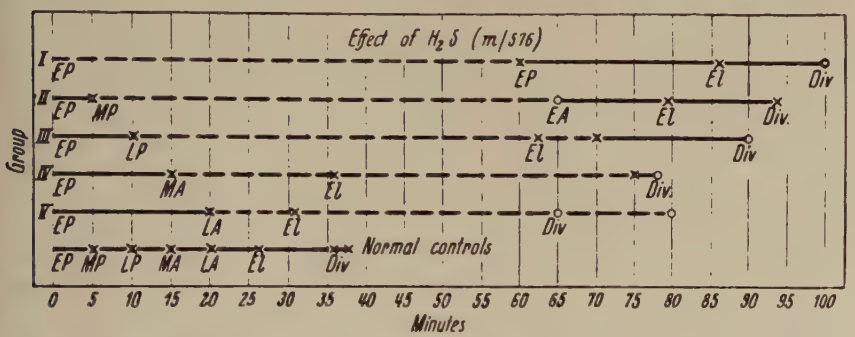

FIGURE 3. Showing the average progress of mitosis and cytokinesis in five groups of amoeba, each exposed for 60 minutes to m/576 H_2S in distilled water, and one group of controls in water alone. Exposure in group I was started as mitosis began. Exposure of groups II, III, IV, and V was started 5, 10, 15, and 20 minutes respectively after the beginning of mitosis. Dotted portions of the graph show time in H_2S. The points marked EP, MP, LP, EA, MA, LA, EL, and Div. designate average stages of fission at times indicated, and are to be read as Early, Mid, and Late Prophase, Early, Mid, and Late Anaphase, Elongation, and Division (of the cell), respectively.

in combination with the observed sequence of events in fission and in its failure, indicates that the sol-gel transformation, functioning much as described by Mast for normal locomotion but initiated in a bi-polar sense, is the most important factor in the process. It is obviously the task of the cytochemist to delve into the energetics of this process, but possibly some ideas in this direction will emerge when the nuclear phase of fission is considered.

Control of Nuclear Fission. Inhibition of nuclear fission of a reversible type is obtained by exposing cells undergoing division to low concentrations (5.6×10^{-3} to 1×10^{-3} of H_2S or NaCN [6]). The process is slowed and the decrease in mitotic velocity is correlated with the phase of the nucleus at which exposure is begun. The greatest slowing is obtained when exposure is begun in early prophase. Nuclei in very late anaphase are hardly slowed. A representative experiment with H_2S is shown in FIGURE 3.

CO in the dark has no effect on mitotic velocity and H_2O_2 does not antagonize the effect of the reductants, NaCN and H_2S. CO_2 however, does inhibit mitosis with production of abundant and occasionally very large

nuclei, suggesting possible polyploidy,[6] but this could not be shown, since, while cells so treated lived and behaved quite normally for some time, they all died without again undergoing fission.

No reagent has thus far been found to raise definitely the velocity of mitosis. It was found possible, however, to hasten or retard the initiation of mitosis.[7]

Hammett, as is well known, has stressed the importance of the SH radical as a stimulant to cell proliferation, and a considerable body of work[8, 9, 10] showed that glutathione had a powerful effect on proliferation in *Amoeba proteus*. How rapidly this reagent acted, or how dependent such activity is on the chemical structure of this and other compounds carrying the radical, was not very clear.

It was found possible to select, under controlled conditions, samples of cells from *Amoeba* cultures which collectively would form populations with a relatively very high rate of entry into mitosis, and to get experimental evidence from these as to the rapidity and pattern of reaction to various sulphur-containing compounds.[7] The type and speed of reaction of the cell to exposure to these compounds varied with their concentration and also with their structure. The results of such studies on the number of cells entering prophase as a function of time when the following reagents were added to the environment in high dilution (2×10^{-4} to 5×10^{-3} mols per liter) may be summarized as follows. Methionine and cystic acid produced no change. Cystine, cysteine glutathione, cystine disulfoxide, B B′-dithiodiproprionic acid, an equi-molar mixture of cysteine, glycine, and glutamic acid (the component amino acids of the tripeptide glutathione) all changed the rate of entry into prophase. There were two classes of effects noted: (1) a saltatory shift in rate, either an increase or decrease, (2) a change from one level of acceleration to another again in either direction (all control rates showed some positive acceleration). The effect of any given reagent was a more or less complex pattern of these two types of change. With some compounds, *e.g.*, cysteine, changes in both directions of both types of change were obtained.

The reaction given by glutathione, an essentially stimulative reagent, could not be duplicated by using a mixture of its component amino acids. This gave a reaction pattern typical of cysteine alone. Cysteine disulfoxide in a freshly made solution was essentially negative. An aged solution (*i.e.*, one that had partly decomposed) gave a temporary depression in rate. Since cysteic acid, an end product, was negative in action, it appears probable that cysteine sulphonic acid, an intermediate product, is responsible for the depressant action. (See Chalkley and Voegtlin[7] for details.)

The picture presented by these complex cell responses strongly suggests that initiation of fission is dependent upon complex intracellular equilibria that are very strongly affected by compounds containing labile sulfur.

Considering the evidence on nuclear fission thus far obtained for *Amoeba proteus*, it would appear from the action of NaCN and H_2S and CO_2 that it is probably activated, at least in part, through oxidative mechanisms. The resistance to CO suggests, however, that such mechanisms are not the

same as those controlling the over-all oxidative metabolism of the cell. Further evidence on this point is to be found in the paper by Voegtlin and Chalkley.[6]

In summary, then, the evidence points to the process of fission in *Amoeba proteus* as depending on its initiatory, *i.e.*, preparatory, phase, and on the attainment of certain intracellular equilibria, in which attainment labile sulfur compounds play a large part. In the mitotic phase (*i.e.*, nuclear fission), the evidence points to the predominance of mechanisms probably not identical with the over-all respiratory mechanism but influenced strongly by certain oxidative inhibitors. In the phase of cytoplasmic fission, the overt mechanism is quite evidently closely allied to and, in great part, appears identical with, that of locomotion, *i.e.*, localized sol-gel change, gel contraction, and adhesion to the substrate by the cell membrane (plasma lemma of Mast), correlated in action to produce the observed changes in material configuration.

It also appears that the stimulus to cytokinesis begins with the breakdown of the nuclear membrane at metaphase and ceases with the reconstruction of the nucleus. In this respect, the sulphydryl cycle, as revealed by the nitroprusside reaction in the cell, is suggestive, consisting as it does of release of sulphydryl-containing material from the nucleus into the cytoplasm at metaphase and gradual increase in such material in the nucleus after its reconstruction.[5]

It must not be overlooked that, in nuclear fission, such sol-gel transformations are also involved in formation of the spindle and in movement of the daughter nuclei in anaphase dissolution of the nuclear membrane, *etc.* It is only when and if these physical changes of state are demonstrably linked to the chemical transformations that accompany them that the phenomena of fission will become clear. It would appear that in many ways active research into the controlling factors of the sol-gel equilibria in the cells, and all that implies, is the direction in which future physiologic study in this field must be pressed.

Bibliography

1. Chalkley, H. W. 1935. The mechanism of cytoplasmic fission in *Amoeba proteus*. Protoplasma **24:** 607–621.
2. Chalkley, H. W. & G. E. Daniel. 1934. The effect of certain chemicals upon the division of the cytoplasm in *Amoeba proteus*, with particular reference to salt antagonism and the interaction of salts and organic acids. Protoplasma **21:** 258–269.
3. Mast, S. O. 1923. Mechanisms of locomotion in amoeba. Proc. Nat. Acad. Sci. **9:** 258–261.
4. Pollack, H. 1927. Micrurgical studies in cell physiology. VI. Calcium ions in living protoplasm. J. Gen. Physiol. **11:** 539.
5. Chalkley, H. W. 1937. The chemistry of cell division. VII. The distribution of sulphydryl in *Amoeba proteus* in interkinesis and mitosis, as shown by the nitroprusside test. Protoplasma **28:** 489–497.
6. Voegtlin, C. & H. W. Chalkley. 1935. The chemistry of cell division. IV. The influence of H_2S, HCN, CO, and some other chemicals on mitosis in *Amoeba proteus*. Protoplasma. **24:** 365–383.
7. Chalkley, H. W. 1942. Effect of certain sulfur-containing compounds on the initiation of mitosis in *Amoeba proteus*. J. Nat. Cancer Inst. **2:** 425–447.
8. Voegtlin, C. & H. W. Chalkley. 1930. The chemistry of cell division. I. The

effect of glutathione on cell division in *Amoeba proteus*. Pub. Health Rep. **45:** 3041–3063.

9. Chalkley, H. W. & C. Voegtlin. 1932. The chemistry of cell division. III. Inhibition of cell divisions in *Amoeba proteus* by high dilutions of copper salts—antagonists of copper and glutathione. Pub. Health Rep. **47:** 535–560.
10. Chalkley, H. W. & C. Voegtlin. 1940. The effect of variation in oxygen tension and sulphydryl concentration on nuclear growth and fission in *Amoeba proteus*. J. Nat. Cancer Inst. **1:** 63–75.
11. Lavine, T. F. 1936. The oxidation of cystine in non-aqueous media. VI. A study of the reactions of the disulfoxide of l-cysteine, especially of its dismutative decomposition. J. Biol. Chem. **113:** 583–597.

MICRURGICAL STUDIES ON THE KINETIC ASPECTS OF CELL DIVISION

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The continuous existence of a close functional relation between the nucleus and the cytoplasm has long been established. For example, the influence of the interkinetic nucleus on protoplasmic growth was shown through an early classic experiment of Gerassimow. By exposing the alga, *Spirogyra*, to cold, he found that the partitioning walls between some of the dividing cells were so affected that both daughter nuclei became incorporated in one of the cells, leaving the other cell non-nucleated. On returning the *Spirogyra* to room temperature, the binucleate cells grew to unusually large dimensions, while the non-nucleated cell remained small.

The interkinetic nucleus has also been found to protect the cell from the cytolytic effects of mechanical injury. This was indicated by micrurgical experiments[1] on fibroblasts in tissue culture. If the nucleus of a mononucleated cell is punctured, complete cytolysis ensues (FIGURE 1). On the other hand, if the same operation is performed on one of the nuclei of a binucleate cell, the cell recovers from the injury (FIGURE 2). The immediate effect of the puncture is the development of an opacity about the injured nucleus and a breakdown of the filamentous mitochondria in the vicinity. The cytoplasm around the untouched nucleus maintains its normal appearance and, after several minutes, the cell recovers completely, except that it is now mononucleated.

Thus far, there has been no clear-cut, visible evidence of material being transferred from the interkinetic nucleus into the cytoplasm. It is otherwise during the periodic intervals when the cell undergoes mitosis.

A universal feature of cell division, other than the increase in volume of the prophase nucleus and the accompanying condensation of the chromosomes, is the loss of morphological identity of the nuclear membrane and, except for the chromosomes, the mixing of nuclear substance from the cytoplasm.

There are indications that the cells, during this period of nuclear and cytoplasmic mixing, are particularly susceptible to externally applied injurious agents. We have already noted the cytolytic effect of artificially inducing a mixture of nucleoplasm with cytoplasm by puncturing the interkinetic nucleus with a microneedle. At the onset of mitosis, the mixing occurs spontaneously, with no resultant cytolysis. However, the critical nature of the period of mixing can be demonstrated by microoperations on the starfish egg. Micropunctures of the cytoplasm, soon after dissolution of the germinal vesicle (prophase nucleus), tend to induce extensive cytolysis. At other times, the egg cytoplasm can be subjected to repeated micropunctures without such cytolysis.

The spontaneous mixing of the karyoplasm with the cytoplasm can be

strikingly demonstrated in maturing starfish eggs which have been injected with phenol red, a pH indicator.[2] The germinal vesicle assumes the pink color of the alkaline range of the indicator, while the cytoplasmic matrix assumes the yellow color of the acid range. When the nuclear membrane breaks down, the cytoplasmic granules can be seen invading the pink nuclear region, while progressively lengthening streams of pink spread from

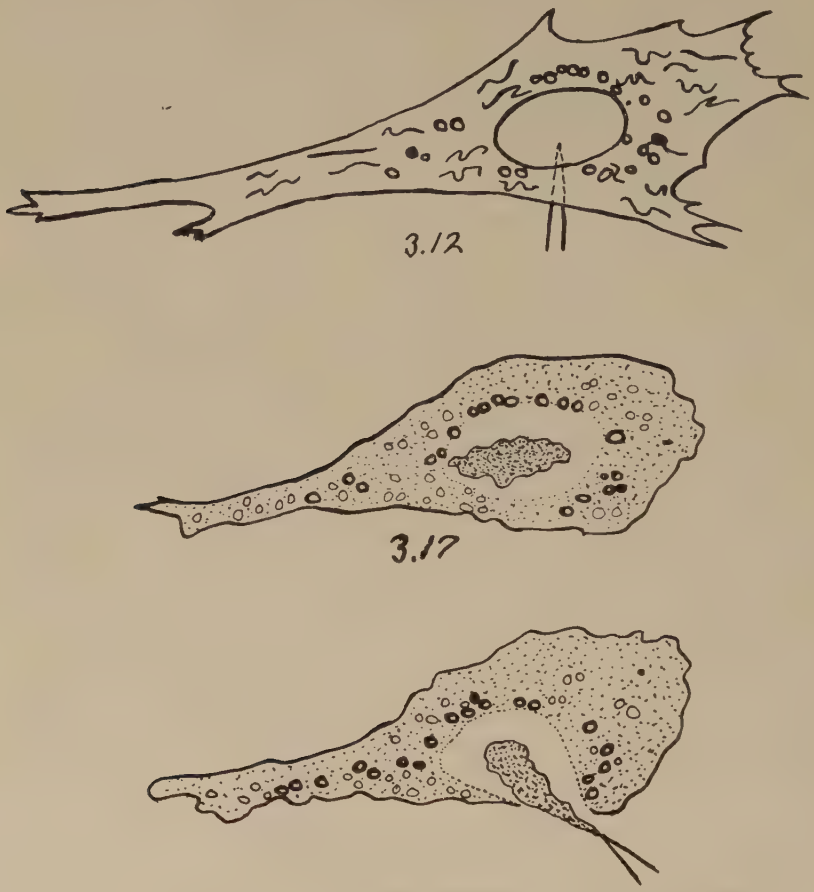

FIGURE 1. Damage induced by micropuncturing the nucleus of a fibroblast in tissue culture

the nuclear region into the yellow cytoplasm. The mixing continues until the entire egg appears reddish orange. After a few seconds, the color reverts to the yellow of the buffered pH of the egg cytoplasm.

All of the karyoplasm must permeate the egg to permit the appearance of asters followed by successful cleavage.[3] If a series of maturing eggs are cut in two at successive stages while the fluid of the germinal vesicle is diffusing into the cytoplasm, the cut-off portion of the egg will, upon fertilization, give evidence of differing amounts of karyoplasm present by the varying abilities of the portions to form asters and to cleave (FIGURE 3).

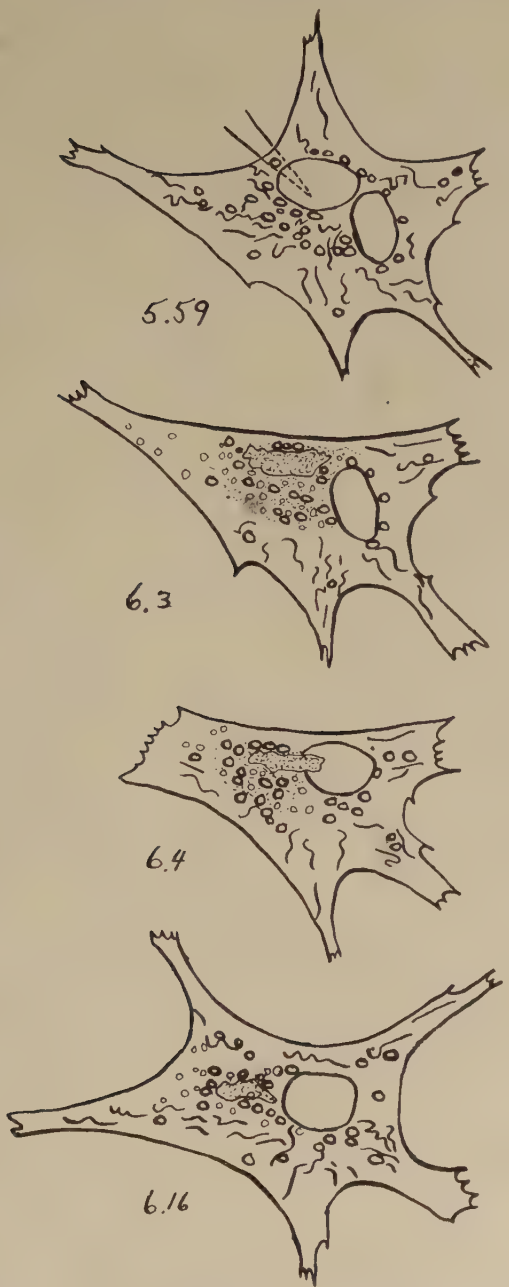

FIGURE 2. Partial and reversible damage induced by puncturing one of two nuclei of a binucleated fibroblast.

The diffusion of the karyoplasm through the cytoplasm must be followed by a period of ripening or aging of the karyoplasm before asters appear.

The ripening does not require the presence of the definitive female nucleus. Starfish eggs were cut in two after the germinal vesicle had disappeared. Both fragments of each egg were inseminated simultaneously and the pairs kept under observation. No aster developed in any of the fragments until the corresponding fragment possessing the female nucleus had formed the second polar body. A sperm aster then appeared in both fragments, indicating a similar length of ripening in both.[4]

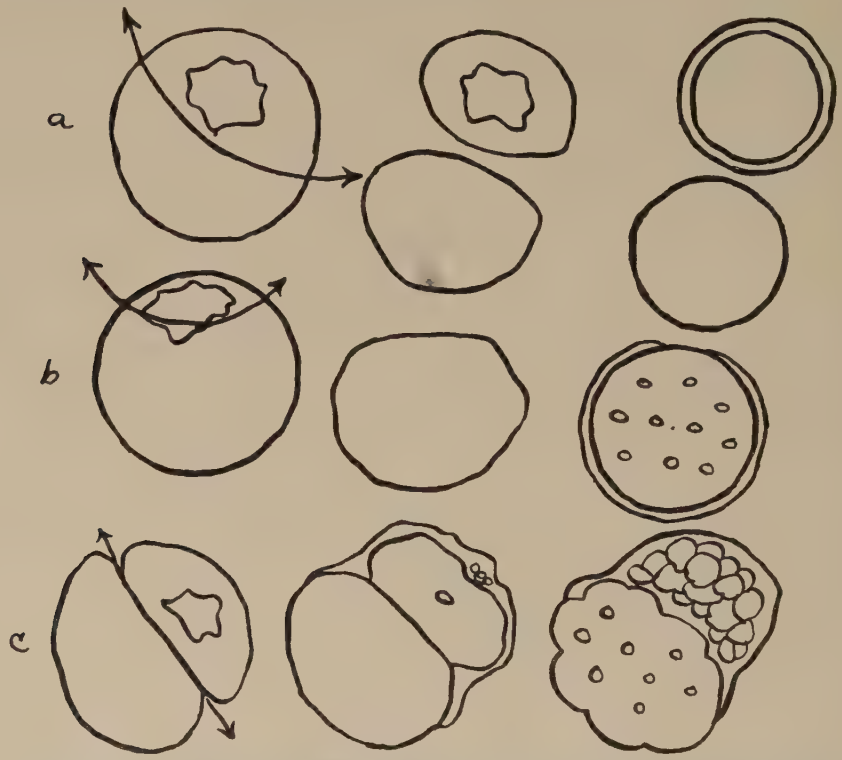

FIGURE 3. Fertilizability by insemination of fragments of starfish eggs cut in two at successive stages of diffusion of the germinal vesicle material. (a) Early stage. Only the nucleated fragment forms a fertilization membrane and undergoes normal cleavage. (b) Later stage. The cut passing through the germinal vesicle caused cytolysis of the nucleated fragment. The other fragment contained sufficient nuclear material to permit activation with successive cleavages of the sperm nucleus but with no cell cleavage. (c) Still later stage. The nucleated fragment was normal and underwent successive cleavage . The non-nucleated fragment underwent cleavages of the sperm nucleus; also, there were attempts at cleavage of the egg.

Kopac[5] has been able to show a difference in physico-chemical behavior between the karyoplasm and the cytoplasm of immature starfish eggs. He has also secured evidence for a similarity in reaction of the karyoplasm of the immature egg with the karyo-cytoplasm of mature eggs. The method used was to note the effect of cytolysis on the rate of spontaneous wrinkling of oil drops injected into the eggs, the wrinkling, termed the Devaux effect, being interpreted as due to denaturation of the protein molecules adsorbed on the oil drop. The experiment was performed on mature and immature starfish eggs centrifuged to displace the cytoplasmic granules to the heavy

pole. The lighter poles of the mature eggs showed a hyaline zone while, in the immature eggs, this zone was occupied either by an intact germinal vesicle, or by the hyaline fluid of a germinal vesicle whose membrane had just faded. Oil drops were injected into the hyaline and granular zones of both types of eggs. It was noted that, in the immature egg, the Devaux effect developed more rapidly in the hyaline than in the granular zone. The reverse was found with the mature egg, namely, that the Devaux effect occurred more rapidly in the granular zone than in the hyaline zone and at about the same rate as in the hyaline zone of the immature egg.

A difference in behavior between the cytoplasm of the immature egg and that of the mature egg had already been noted by Delage, Wilson, and Yatsu and discussed extensively by Mathews,[6] with many significant references. However, no term has ever been introduced to distinguish the two types of cytoplasm. It is proposed that the cytoplasm into which the karyoplasm of the prophase nucleus has diffused be termed karyo-cytoplasm.

In general, the chief difference between the karyo-cytoplasm of the maturing egg and of a somatic cell in prophase is that the prophase mature egg requires a special stimulus to induce cleavage, while the prophasic somatic cell does not. The blastomeres, subsequent to fertilization of the egg, undergo periodic successive divisions similar to those of somatic cells. The relatively brief intervals between the successive cleavages of the egg, in contrast to the longer intervals in the cells of normal somatic tissues, may possibly be related to the peculiarly long period of the karyo-cytoplasmic "aging" in the egg. Is it possible that constantly recurring cell divisions in tissues, such as malignant tumors, are related in some way to the type of intermixing of the nuclear nucleoproteins with the somatic cytoplasm, as appears to occur in the maturing egg?

Once mitosis has been initiated, there occurs a series of physical phenomena which culminate in the division of the cells. The most obvious of these may be grouped under several categories, as follows: (1) changes in the extraneous material enveloping the cell; (2) tension changes at the bounding surface; (3) changes in the protoplasmic surface layer and the underlying cortex; (4) changes in the mitotic spindle and the cell in mitosis; (5) the gelation of the forming aster and streaming movements of the cytoplasm; (6) the gelated state of the cortex; (7) structural and kinetic characteristics revealed by transecting the egg diagonally to the cleavage plane; (8) changes of the protoplasmic surface of the cleaving furrow; and, (9) the persistence of a joining strand after cleavage.

(1) *Changes in the Extraneous Material Enveloping the Cell.* Animal cells about to divide, particularly epithelial cells, tend to draw away from their neighboring cells. This indicates that the cement which binds them together has become loosened. The cement is an extraneous coat secreted by the cells and depends for its maintenance upon the presence of calcium in the medium. The cement can be weakened either by a deficiency of the calcium or by a condition, such as a lowered pH, which increases solubility of the calcium in the cement.[7] The latter could occur by the liberation of increased CO_2 through increased metabolic activity of the cells.

(2) *Tension Changes at the Bounding Surface.* It is well known that a

cell in prophase tends to become round. This suggests an increased tension at its surface. Columnar, cuboidal, and pavement-like epithelial cells, during interkinesis, seem to be gelated, plastic bodies. When these cells pass into prophase they become solated and assume a shape approaching that of a sphere as their equilibrium figure. These cells press on their more plastic neighbors and deform them.

In the case of fibroblasts and wandering cells, the consequence of solation and the development of a tension at the surface is the withdrawal of their extended processes and pseudopodia. The solation occurs primarily at the base of the processes, so that the shortening occurs while the tips of the processes still remain attenuated and relatively stiff.

(3) *Changes in the Protoplasmic Surface Layer and the Underlying Cortex.* The rounding of the cells is accompanied by the development of a gelated cortex which underlies a fluid protoplasmic surface layer. The shortening of the attenuated pseudopodia of fibroblasts in tissue culture may exhibit temporary elevations on the surface, as if the shrinkage of the underlying cortex was too rapid to allow the surface layer to reduce its area concomitantly. The elevations eventually flatten out. Similar elevations appear on the withdrawing prolongations of pseudopodial cells which are becoming round preparatory to mitosis.[23] As the prophase stage progesses into the metaphase and anaphase, the gelated cortex of the cell also undergoes a shrinkage, which spreads in a wave from the poles but does not involve the incipient cleavage furrow at the equator of the cell. This shrinkage may in part explain the appearance of the temporary, bleb-like elevations which was first noted by Strangeways[8] and which has been so prominently displayed in motion pictures of dividing cells in tissue culture. Barber and Callan[9] have suggested that the "bubbling" is related to the intake of liquid which they noted as occurring during anaphase in the epidermal cells of the newt. It is possible that the initital elevations at the poles are due to shrinkage and that the subsequent more prominent elevations are involved in the uptake of liquid.

Dan and coworkers[10] drew some interesting deductions from their observations on the movements of kaolin particles adhering to the surface of naked sea urchin eggs. As the egg elongated prior to cleavage, they observed that the particles over the poles spread apart, while those near the incipient cleavage plane moved closer together, indicating shrinkage. They were able to relate these movements to similar but more delayed ones of the pigment granules imbedded in the gelated cortex of the egg. There would thus appear to be a general movement of the protoplasmic surface film and of the underlying cortex which is directed from the poles toward the equator. An ingenious method of detecting movements of the cortex to the equatorial region is that of Dan,[11] who produced microperforations in the cortex in the region of the incipient cleavage furrow. The perforations, at first circular, became elongated as their walls were drawn into the equator (FIGURE 4). The cortex at the poles thins out and weakens. The weakening at the poles can be demonstrated also in the dividing spermatocyte of the grasshopper, *Dissosteira*, where agitating the polar surfaces with

microneedles readily induces protrusions while, by contrast, the rest of the surface exhibits a surprising stiffness.

There seems to be a relation between the peculiar conditions at the poles of the cell and the approach to them of the chromosomal plates or of the daughter nuclei during anaphase and telophase. It is at this time that the surface and cortical streaming from the poles to the equator occurs.

In somatic cells, the major part of the cell is occupied by the mitotic spindle. The chromosomal plates approach the poles of the cell during anaphase at an earlier stage of mitosis than do the echinoderm eggs.

(4) *Changes in the Mitotic Spindle and the Cell in Mitosis.* The mitotic spindle is being considered here only in regard to its general physical state and to its relation with the process of cell division.

The dynamics of the spindle in regard to the movements of the chromosomes is still in the realm of speculation (*e.g.*, the discussion of Bernal[12] on

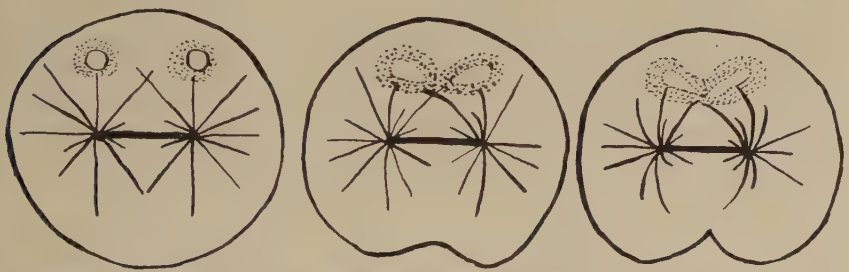

FIGURE 4 (from Dan). Two persisting perforations were made with a microneedle through the egg in the region of the future cleavage plane. (a) Immediately after making the perforations. (b) and (c) Successive deformations of the perforations as a result of cortical flow into the equator.

tactoids and the tactoid structure of the spindle). The spindle possesses an appreciable degree of solidity. This was early indicated in experiments by Foot and Strobell,[13] who found that the spindle of the first maturation retained its form after flowing out of punctured eggs of *Allolobophora foetida.*

In grasshopper germ cells during metaphase, it is a gel which readily reverses to a sol by thrusts with the tip of a microneedle. Thereupon, the chromosomes fall out of their orderly arrangement at the equator and can be readily isolated.[14, 15] The gel to sol reversibility of the polar part of the spindle persists as long as it is evident during anaphase. On the other hand, the lengthening, interzonal portion between the separating chromosome plates is much more stable and firm to the touch. It maintains its relatively high consistency even while it is being dragged out of the cell with the chromosomes adhering to its two ends. Bělař's experiments[16] indicate a similar high viscosity for the interzonal portion. He found that immersion in hypertonic sucrose of *Tradescantia* stamen hairs in mitosis causes the interzonal portion to become narrowed and greatly elongated. Microdissection experiments on *Rhoeo* pollen mother cells, grasshopper germ cells, and stamen hair cells give evidence of a pronounced difference in consistency between the polar and the interzonal portions of the spindle. During their advance to the poles, the chromosomes appear to be moving into a delicately

constructed, relatively fluid region, while behind them they leave an ever-lengthening, relatively solid column of material. Schmidt[17] has demonstrated with the polarizing microscope that the polar region of the spindle is birefringent, while the interzonal region is not. The anisotropic condition of the polar spindle suggests an orientation of materials which may serve to direct movements of the chromosomes. The isotropic material of the interzonal portion may be a spontaneously accumulating substance between the chromosome pairs. Such an explanation for the separation of the chromosomes has recently again been advocated.[18] Incidentally, an argument against the interpretation that the fibers seen in the spindle in fixed material represent fibers in the living state was presented long ago by Foot and Strobell.[13] These observers noted that the chromosomes in the spindles of the eggs with which they worked were sometimes massed in one half of the spindle. In spite of this, on fixation, the spindle was not distorted and the rays in it were present and extended from pole to pole as usual.

An interesting feature brought out by Barber and Callan,[9] in their studies on mitosis of the epidermal cells of the newt, is the swelling of the cells during anaphase and telophase. Prior to anaphase, the cell-outlines are polygonal. During anaphase, the cell swells and forms an even, rounded outline at the expense of the neighboring cells. From this, they inferred an uptake of liquid as being due to an intracellular increase of osmotically active material.

There is a possibility that a similar explanation may be given to the findings of Stern[19] in his studies of the effect of hypertonic sucrose (1.5) on the pollen mother cells of *Trillium*. Stern found that the plasmolytic action of the sucrose solution, which is effective at other stages, was evanescent or even absent on cells during very late prophase and metaphase. He explained the phenomenon as an increased permeability to sucrose. However, the phenomenon might be due to an increased metabolic activity in releasing osmotically active material in the cell sufficient to counteract the plasmolytic effect of the sucrose. There may also be cited observations on the fertilized eggs of Echinoderms. Chambers[3] had noted that starfish eggs undergo a spontaneous increase in diameter not later than ten minutes after fertilization. Shapiro[20] has presented similar evidence for the *Arbacia* egg, in which he finds an increase of 2.7 per cent in volume within 25 minutes after fertilization. At about this time, the *Arbacia* egg is in the "streak stage," during which the metaphase is passing into the anaphase and telophase of mitosis. The fact that metabolic activity does cause an increase in osmotic pressure with increased swelling has been shown for muscle. Hill and Kupalov[21] discuss this by referring to earlier investigators. The question regarding muscular activity is still open as to whether the increased osmotic pressure is due to accumulation of material between, or within, the individual fibers.

(5) *The Gelation of the Forming Aster and Streaming Movements of the Cytoplasm.* The monaster, a gelated body with numerous radiations about a central hyaline zone, has been studied in echinoderm eggs which have been fertilized either by sperm entry or by artificial activation, irrespective of the presence[23] or absence[24] of female pronuclei. The hyaline central region of the monaster normally contains the male and female pronunclei. The

presence of either or both pronuclei appears to be incidental since, in artificial parthenogenesis, the male pronucleus is absent and, in parthenogenetic merogony, both male and female pronuclei are absent.

The monaster progressively grows in size until it occupies most of the egg. The central, hyaline region, called the centrosphere, also increases and, by microdissection, has been shown to be a lake of fluid enclosed by the gelated material of the aster, the radiations of the aster being delicate paths of centripetally streaming fluid. Fol,[25] who first proposed the term, aster, already had presented the idea that the radiations are due to streams of centripetal flow. The following is quoted from him (page 194) "*Il semble donc admissible que les lignes claire de l'aster ne sont en réalité que des courants de sarcode qui viendraient confluer en un amas central.*" After the monaster has increased to its maximum, its radiated appearance fades out as the gelated state reverts to the sol. However, the hyaline liquid centrosphere

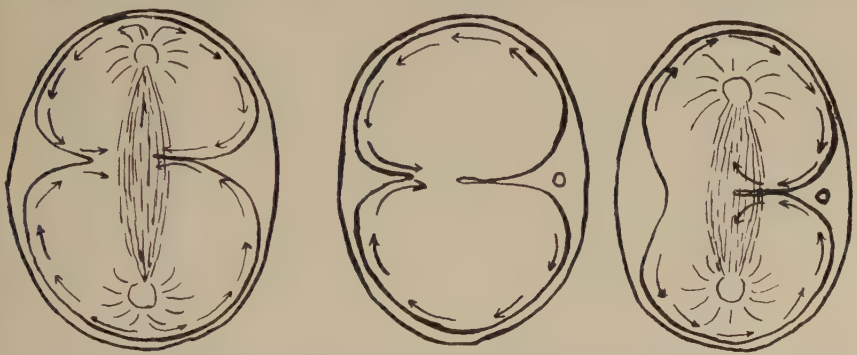

FIGURE 5. (from Spek). Peripheral flow of cytoplasm (indicated by arrows) in cleaving nematode egg. (a) and (b) The flow may form asymmetrically, *i.e.*, the left side predominating, as in (a), and one minute later a reversal of peripheral flow accompanied a deepening of the furrow on the right side, as in (b). (c) A symmetrically cleaving furrow.

persists and spreads out to collect into two lake-like centrospheres. New astral radiations appear around these to constitute the typical amphiaster of the egg on its way to cleavage.

An interpretation of the amphiastral configuration, suggested long ago by Heidenhain, has been recently restated by Dan[26] with the support of some experimental evidence. It was considered that the astral rays serve as guy ropes which exert a pull on the sub-polar and equatorial surfaces of the egg cortex in such a way as to induce the equatorial constriction of the cleavage furrow. This interpretation is difficult to accept. For example, there are indications that the peripheral ends of the astral radiations fade into a fluid zone immediately underlying the gelated cortex. Moreover, the aster can be artificially rotated without affecting the contour of the egg.

Another explanation is that the approach of the daughter nuclei to the poles gives rise, and that the amphiastral figure gives direction, to the phenomenon of streaming which J. Loeb[27] postulated as underlying cell cleavage. This was emphasized by Spek in his studies on the cleavage of the nematode egg[28] (FIGURE 5.). The two global, gelated asters at the poles of the mitotic spindle may serve to give a curved sweep to the currents of flow

which emanate from the two opposite poles of the cell. The surface layer, the cortex, and the underlying peripheral fluid cytoplasm are involved in the superficial flow around the asters toward the center at the equator of the cell. The possibility of a dead space in the equatorial region where the two opposite vortices meet is suggested by a striking experiment[29, 30] in which a drop of oil was injected into a fertilized *Lytechinus* egg. During mitosis, the drop moved in the cytoplasm to the equatorial region, where it remained until it was pinched in two by the advancing cleavage furrow. By the time the egg had reached the 8-celled stage, each blastomere contained an oil drop which had resulted from successive divisions of the drop originally introduced.

The sea urchin egg remains spherical during the earlier period of mitosis with its relatively short spindle. The currents from the polar regions of the cell do not start until the two daughter nuclei have been formed and are as far apart as they can be carried by the lengthening spindle. Peripheral

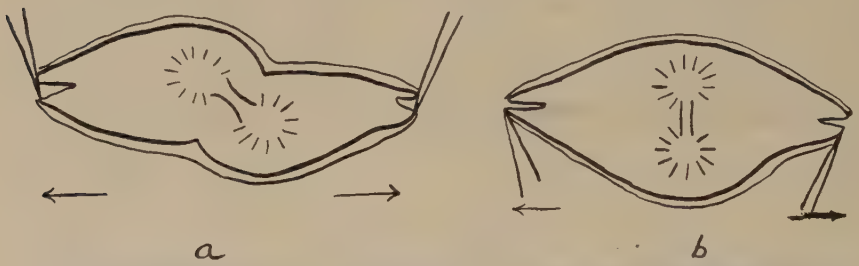

FIGURE 6. Cleaving sea urchin egg. (a) Stretching the egg diagonally to the cleavage plane and holding the egg in this distorted state had no effect on the plane of the original furrow. (b) Stretching the egg parallel to the cleavage plane resisted formation of the cleavage furrow. (A small portion of the cell cortex was torn at the points where the needles were applied.)

cytoplasmic streaming from the poles then begins and the cell elongates, possibly assisted by the lengthening of the interzonal portion of the spindle, whereupon the furrow appears which cleaves the egg in two.

The fact that the approach of a nucleus to the surface of a cell does create surface movements can be experimentally demonstrated in *Amoeba dubia*. One way of doing this is to impale the nucleus on the tip of a vertically placed microneedle. The Amoeba flows along mainly in one direction until the nucleus, held stationary in this way, comes to lie close to one surface of the *Amoeba*. Thereupon, pseudopodial elevations develop on the surface in the immediate vicinity of the nucleus and the *Amoeba* reverses its flow. This back and forth movement of the *Amoeba* may be continued for hours, as long as the impaled nucleus is not injured by sudden, vibrational movements of the microneedle.

In the sea urchin egg, the cleavage furrow persists along its original path as long as the amphiaster is intact and the proper directional streaming continues.[23] This is shown in FIGURE 6. In 6a, the egg was stretched diagonally to the long axis of its amphiaster. The position and integrity of the amphiaster were not interfered with and the original direction of the cur-

rents persisted. The cleavage furrow continued along its original plane. In 6b, the stretching was done at right angles to the long axis of the amphiaster. The latter persisted but the streaming pattern was so interfered with that the cleavage furrow failed to form.

(6) *The Gelated State of the Cortex.* The asters in the fertilized echinoderm egg are easily and reversibly solated by thrusts of the microneedle. The gel of the cortex, however, is much more resistant and will maintain itself for some time, even though a part of it be destroyed by tearing through the surface of the egg.[30] A fertilized *Arbacia* egg, freed of its extraneous coats, was mounted in a drop of calcium-free sea water. When the cleavage furrow was well advanced, one pole of the dumb-bell shaped egg was torn with a microneedle. The astral configuration of the torn incipient blastomere

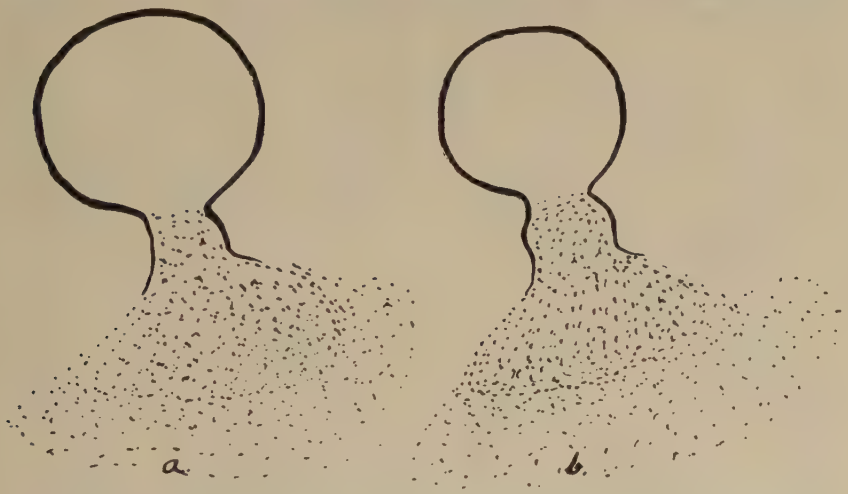

FIGURE 7. Cleaving sea urchin egg in calcium-free sea water with one of the incipient blastomeres torn by means of a microneedle. (a) The tear caused a disintegration of the torn blastomere, leaving the floor of the walls of original cleavage furrow as a persisting stalk connected with the remaining blastomere. (b) The contents of the remaining blastomere flowed out through the stalk, resulting in a progressive decrease in spherical surface of the blastomere.

disappeared and the granular contents scattered, while a wave of disintegration spread rapidly over the surface but with increasing delay as it approached the wall of the cleavage furrow. A few seconds later, the aster, in the untorn incipient glastomere, faded from view, while the blastomere diminished in size as its contents poured through the stalk into the surrounding medium. Two significant features were noted: the wall of the stalk retained its original dimensions and the contour of the blastomere remained smooth (FIGURE 7). Sichel and Burton[31] made successive measurements of the diameter of the decreasing blastomere from a motion picture film. The progressive slowing in the rate of decrease led them to conclude that the cortex exhibited elastic tension.

(7) *Structural and Kinetic Characteristics of the Egg Demonstrated by Transecting the Eggs Diagonally to the Cleavage Plane.* The localized growth of a gelated cortex in the region of the cleavage furrow has been presented by

Schechtman.[32] That the movements along the furrow are related to gelation was indicated some time ago by diagonally transecting sea urchin eggs during cleavage.[33] These experiments gave striking evidence for the gelated state of the cortex of the advancing furrow and the separateness of the gelated protoplasmic bodies surrounding the nuclei of the two incipient blastomeres.

The cutting process consists of pressing a horizontally placed shaft of a microneedle down through the egg. The highly fluid state of these eggs and

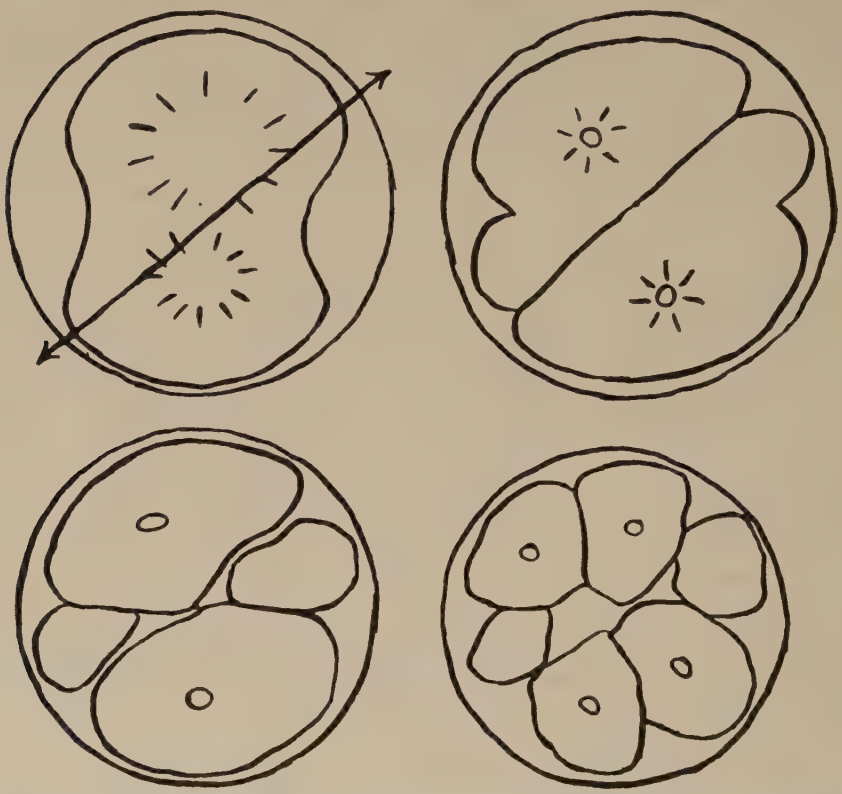

FIGURE 8. (a) Transecting a cleaving sea urchin egg without disturbing the amphiaster. (b) The cleavage furrow continued along the original plane. (c) Completion of the cleavage furrow converted the egg into two nucleated and two non-nucleated portions. (d) The nucleated portions underwent the second cleavage.

the cohesiveness of their cortices permits the pressure to carry the cortex ahead of the shaft of the needle until the shaft passes through the substance of the egg. The egg is then cut through. It is possible to make a diagonal cut through the egg without destroying the gelated state of the dividing egg (FIGURE 8). When this is done, the original furrow continues to advance and cuts off a non-nucleated portion. The nucleated parts eventually undergo cleavage, converting the egg into four normal appearing blastomeres and two non-nucleated fragments.

On the other hand, if the transection is done roughly, both asters of the

amphiaster solate (FIGURE 9). Streaming ceases, the furrow disappears, and the two portions produced by the cut take on the appearance of two completely separated blastomeres. These, eventually, undergo cleavage, converting the egg into four normal appearing blastomeres.

(8) *Changes of the Protoplasmic Surface of the Cleaving Furrow.* When the cleaving egg is in its normal environment of sea water, and is invested

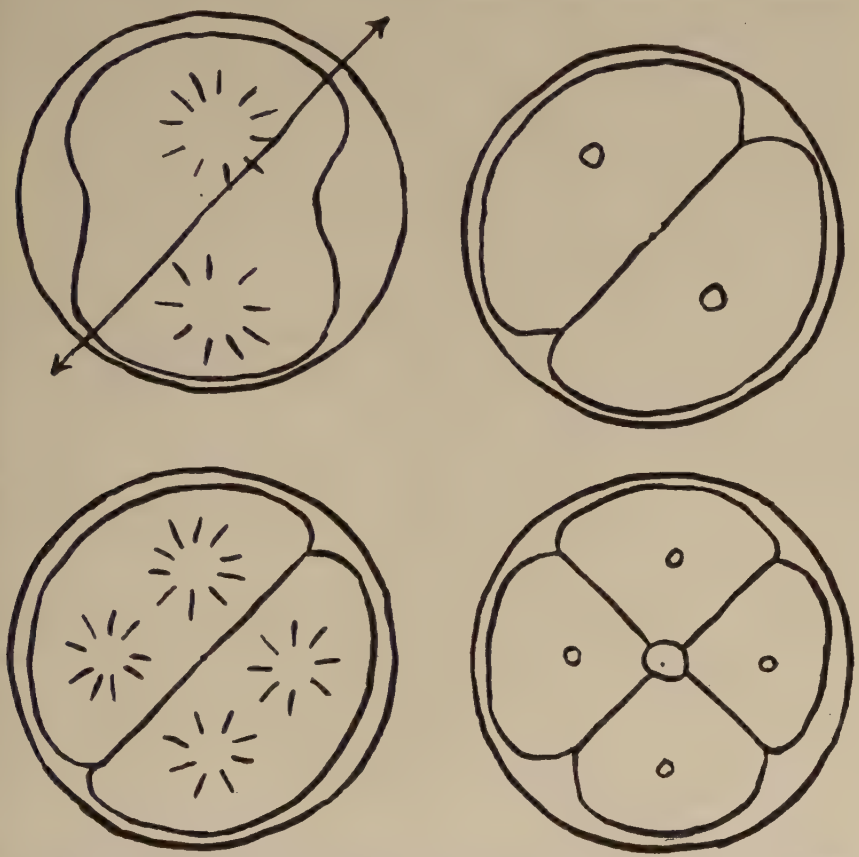

FIGURE 9. (a) Transecting a cleaving egg in such a manner as to destroy the amphiaster. (b) The original cleavage furrow disappeared and the nuclei of each portion moved into central positions. (c) New amphiasters appeared. (d) The second cleavage converted the egg into four normal appearing blastomeres.

with its usual extraneous coats, the walls of the two incipient blastomeres on opposite sides of the advancing cleavage furrow become closely pressed together, except in the trough at the tip of the deepening furrow. Here, there is an appreciable space, tear-drop shaped in cross section, separating the protoplasmic surface of the newly forming walls (FIGURE 10). This space is occupied by a substance which, in the older part of the furrow, is flattened out and serves as an extraneous coat, the so-called hyaline layer, which keeps the protoplasmic surfaces of the furrow from direct contact.

No such coat forms if the cleaving eggs are immersed in calcium-free sea

water. Successful cleavage then occurs only when the forming blastomeres are free to move apart so that the surfaces of the cleavage furrow do not come into contact. A significant experiment regarding this involves placing the eggs, denuded of their fertilization membranes, on a glass slide containing grooves into which the eggs fit tightly. A coverslip holds the eggs in the grooves. The eggs which form mitotic spindles parallel to the long axis of the grooves are free to elongate to form spherical blastomeres and successful cleavage ensues. Those which have the long axes of their mitotic spindles at right angles to the long axes of the grooves do not form blastomeres.[34] In these eggs, the cleavage furrow begins to form but, as it deepens, the newly forming protoplasmic surfaces of the furrow wall come into direct contact and, there being no intervening extraneous coat, the protoplasmic surfaces coalesce. Thereupon, the incipient furrow disappears. The attempt to cleave is repeated several times as long as the currents of flow continue within the egg. The ease of coalescence of the protoplasmic surface film suggests a high fluidity.

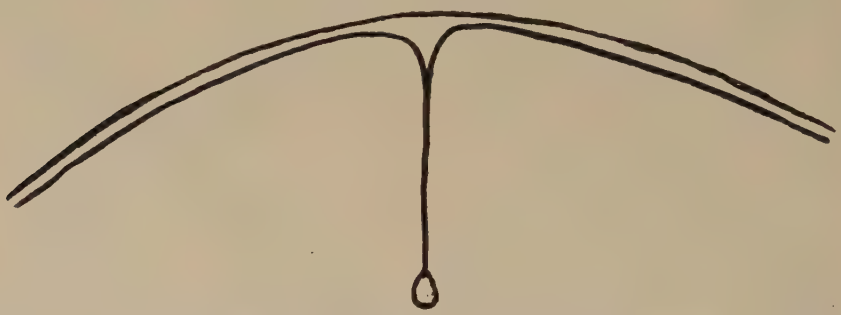

FIGURE 10. Sketch of advancing furrow of a cleaving sea urchin egg under compression in sea water. Note closely appressed walls of furrow with tear-drop contour of advancing tip.

(9) *The Persistence of a Joining Strand After Cleavage.* A phenomenon which has been noted in a wide variety of cells is that the two daughter cells remain connected by a narrow strand. Evidently, the forces which induce the formation of the cleaving furrow do not carry the cell division to completion. In the *Arbacia* egg, there remains a narrow cylinder of cytoplasm sheathed by an extraneous material. The cylinder eventually breaks into one or more beads within the sheath, thereby severing cytoplasmic continuity between the blastomeres. The isolated beads remain as discarded bits of cytoplasm, while the proximal portions of the cylinder flow into their respective blastomeres. The connecting sheath which remains is relatively rigid and persists until the movements of the daughter cells break it mechanically.

Summary

The evidence, as presented in this paper, gives the following conception of some of the mechanisms involved in cell division. There is a continuous interaction of the cytoplasm with the nucleus. Just prior to mitosis, the

interaction between karyoplasm and cytoplasm is very pronounced and is followed by a chain of events which terminates in the division of the cell. First, the cell tends to become round and isolated. Sometime during this period, a swelling of the cell may occur which seems to be associated with an increased, internal osmosis due to an increase in metabolic activity. Then, from the karyo-cytoplasm, there separates out a hyaline, fluid material very evident in the formation of the sperm aster in echinoderm eggs and which is possibly related to the centrosphere familiar to the cytologist. This material collects at the two opposite poles of the mitotic spindle, so that two centrospheres result and the cell exhibits a longitudinal axis. Thereupon, currents flow from the two poles of the cell involving the protoplasmic surface layer, the cortex, and the subjacent fluid region. These currents sweep over the subpolar regions around the gelated bodies or asters and flow deep into the egg at the equator. The currents, accompanied by an advancing gelation of the cortex at the equator, produce a deepening furrow between the incipient daughter cells. The fluid protoplasmic surface layers of the two opposite walls of the developing furrow are prevented from fusing, provided there is sufficient calcium in the medium to stiffen the cementing substance which is secreted by the egg. The substance serves as an intercellular cement. The forces which create the cleavage furrow do not carry the separation of the daughter cells to completion. A connecting column of fluid cytoplasm persists, eventually breaking into beads, whereupon continuity of the two daughter cells is finally broken.

Bibliography

1. Chambers, R. & H. B. Fell. 1931. Microoperations on cells in tissue cultures. Proc. Roy. Soc. B. **109:** 381.
2. Chambers, R. & H. Pollack. 1927. Micrurgical studies in cell physiology. IV. Colorimetric determination of the nuclear and cytoplasmic pH in the starfish egg. J. Gen. Physiol. **10:** 739.
3. Chambers, R. 1921. Microdissection studies, III. Some problems in the maturation and fertilization of the Echinoderm egg. Biol. Bull. **41:** 318.
4. Chambers, R. & E. L. Chambers. 1940. Interrelations between egg-nucleus and cytoplasm of cells and cell fragments. Biol. Bull. **79:** 340.
5. Kopac, M. J. 1948. The surface chemical properties of cytoplasmic proteins. Ann. N. Y. Acad. Sci. **50** (8) : 870–909.
6. Mathews, A. P. 1907. A contribution to the chemistry of cell division, maturation and fertilization. Amer. J. Physiol. **18:** 89.
7. Chambers, R. & B. W. Zweifach. 1940. Capillary endothelial cement in relation to permeability. J. Cell. Comp. Physiol. **15:** 255.
8. Strangeways, T. S. P. 1922. Observations on the changes seen in living cells during growth and division. Proc. Roy. Soc. B. **94:** 137.
9. Barber, H. N. & H. G. Callan. 1943. The effects of cold and colchicene on mitosis in the newt. Proc. Roy. Soc. B. **131:** 258.
10. Dan, K., T. Yanagita, & M. Sugiyama. 1937. Behavior of the cell surface during cleavage. I. Protoplasma **28:** 66.
11. Dan, K. 1943. Behavior of the cell surface during cleavage. V. Perforation experiment. J. Fac. Sci., Tokyo Imp. Univ. Ser. IV, **6:** 297.
12. Bernal, J. D. 1940. Structural units in cellular physiology in the cell and protoplasm. Publication of the Amer. Assoc. Adv. Sci. (14): 199.
13. Foot, K. & E. C. Strobell. 1905. Prophases and metaphases in the first maturation spindle of *Allolobophora foetida*. Am. J. Anat. **4:** 199.
14. Chambers, R. 1915. Microdissection studies on the germ cell. Science, N. S. **41:** 290.
15. Chambers, R. & H. C. Sands. 1923. A dissection of the chromosomes in the pollen mother cells of *Tradescantia virginica* L. J. Gen. Physiol. **5:** 815.

16. Bělař, K. 1929. Beiträge zur Kausalanalyse der Mitose. II. Arch. f. Entwicklungsmech. **118:** 359.
17. Schmidt, W. J. 1939. Doppelbrechung der Kernspindel und Zugfasertheorie der Chromosomenbewegung. Chromosoma **1:** 238.
18. Berkley, E. 1948. Spindle development and behavior in the giant ameba. Biol. Bull. **94:** 169.
19. Stern, H. 1946. The permeability of cells undergoing nuclear division. Trans. Roy. Soc. Canada, Sect. 5, Ser. **3:** 141.
20. Shapiro, H. 1948. The change in osmotically inactive fraction produced by cell activation. J. Gen. Physiol. **32:** 43.
21. Hill, A. V. & P. S. Kupalov. 1931. The vapour pressure of muscle. Proc. Roy. Soc. B. **106:** 445.
22. Chambers, R. 1917. Microdissection studies II. The cell aster: A reversible gelation phenomenon. J. Exp. Zool. **23:** 483.
23. Chambers, R. 1921. The formation of the aster in artificial parthenogenesis. J. Gen. Physiol. **4:** 33.
24. Harvey, E. B. 1940. A comparison of the development of nucleate and non-nucleate eggs of *Arbacia punctulata*. Biol. Bull. **79:** 166.
25. Fol, H. 1879. Recherches sur la fecondation et la commencement de l'henegonie chez divers animaux. Mém. Soc. Phys. et d'Hist. Nat. de Genève **26:** 89.
26. Dan, K. 1943. Behavior of the cell surface during cleavage. VI. On the mechanism of cell division. J. Fac. Sci. Tokyo Imp. Univ. Sec. 4, **6:** 323.
27. Loeb, J. 1906. Dynamics of Living Matter : 65. Columbia Univ. Press. New York.
28. Spek, J. 1938. Oberflächenspannungsdifferenz als eine Ursache der Zellteilung. Arch. f. Entw.-mech. **44:** 5.
29. Chambers, R. & M. J. Kopac. 1937. The coalescence of sea urchin eggs with oil drops. Ann. Rep. Tortugas Lab., Carn. Instit. Wash. Yr. Bk. **36:** 88.
30. Chambers, R. 1938. Structural and kinetic aspects of cell division. J. Cell. Comp. Physiol. **12:** 149.
31. Sichel, F. J. M. & A. C. Burton. 1938. A kinetic method of studying surface forces in the egg of *Arbacia*. Biol. Bull. **71:** 397.
32. Schechtman, A. M. 1937. Localized cortical growth as the immediate cause of cell division. Science **85:** 222.
33. Chambers, R. 1919. Changes in protoplasmic consistency and their relation to cell division. J. Gen. Physiol. **2:** 49.
34. Chambers, R. 1946. Karyokinetic lengthening and cleavage. Anat. Rec. **94:** 373.

THE ACTION OF HYDROSTATIC PRESSURE ON CELL DIVISION

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The pressure sustained by land-dwelling animals seldom exceeds one atmosphere (14.7 lbs/in.^{2}), and, aside from indirect effects upon the concentration of the atmospheric gases in the protoplasm and body fluids, pressure variations within this small range are not of very great physiological importance. Many marine organisms, however, live at depths approaching five miles, where the pressure ranges up to about 15,000 lbs/in.^{2}, and pressures of this magnitude are known to have direct and tangible effects upon the protoplasmic reactions.

Pressure studies on the protoplasmic system have not been very numerous, but, recently, the sol $\rightleftarrows$ gel reaction, which is known to occur in many, if not all, cells, has received considerable attention (Marsland, 1942). Typically, the cortical protoplasm, immediately subjacent to the cell membranes, displays the properties of a gel of greater or lesser tensile strength; whereas, the deeper protoplasm, aside from specific gelated regions, has the consistency of a fairly fluid sol. When high pressure is applied, gelated parts of the protoplasm undergo solation, and, in the fluid state, the protoplasm loses its contractile properties. Thus, pressure exerts a paralyzing effect upon various cells, and pressure provides a tool for analyzing the motility of dividing cells, as well as amoeboid and other types of protoplasmic movement.

Special Methods. It is important to realize that the pressure used in the experiments is of the hydrostatic type. Each cell or tissue is completely surrounded by a liquid medium, and this medium serves to transmit the pressure equally in all directions. This condition eliminates all distortional injury, such as would result if the tissues were compressed locally between impinging solid surfaces. The difference is well illustrated by experiments on dividing *Arbacia* eggs. When these cells are compressed between a slide and coverslip, less than 5 lbs/in.^{2} suffices to cause considerable distortion and to block the cleavage. Under hydrostatic compression, however, the form of the egg remains unchanged, aside from a very slight loss of volume, and the capacity to furrow is not abolished until a pressure of about 5,000 lbs/in.^{2} is reached.

Another important experimental condition is the absence of any gas phase in the system. Many of the early experiments in the field were complicated by the fact that the pressure was applied through the medium of a supernatant atmosphere. This made it difficult to distinguish between the direct effects of pressure and the indirect effects of driving extra quantities of the atmospheric gases into solution in the protoplasm and the surrounding liquid medium. When the gaseous phase is eliminated, the main effect of the pressure must be mediated through small changes in the protoplasmic volume and through the consequent alterations in the fundamental molecular pattern of the protoplasmic system.

The microscope-pressure chamber described by Marsland and Brown (1936) permits cells to be studied at pressures ranging up to 15,000 lbs/in.^{2} In this chamber, specimens may be viewed during the period of compression at magnifications up to 600 diameters. Upper and lower windows, 3 mm. thick, permit light to be transmitted through the chamber to a special objective which, despite an unusually great working distance of 15 mm., possesses a magnification of 30 diameters. Since the specimens in the chamber tend to drop to the surface of the lower window, the bomb is used with an inverted microscope, and good images are obtained with oculars up to 20×.

Measurements of the strength of the gel structure as a function of pressure were made by the centrifugal method, using the centrifuge-pressure bomb devised by Brown (1934). In this apparatus, the cells may be centrifuged while the compression is maintained at any level up to 15,000 lbs/in.^{2} The bomb is divided into two parts: (1) the experimental, or pressure chamber; and (2) the control chamber. A needle valve seals the pressure into the experimental chamber during the period of centrifugation, and, since the centrifugal radius is the same for both chambers, the control (atmospheric) and the high pressure specimens are subjected to an equal centrifugal force.

Nature of Protoplasmic Gel Systems. On the basis of Freundlich's work, three types of gel may be distinguished (Freundlich, 1937). In Type I (exemplified by gelatin), the gelation reaction is exothermic and is accompanied by a diminution of volume (−DV). Such systems should gelate with increasing pressure (or with decreasing temperature) and recent measurements of the pressure effect have substantiated the Freundlich prediction (Marsland and Brown, 1942). In Type II (exemplified by purely thixotropic gels like sodium cleate), the sol-gel reaction is essentially isothermic, with little or no change of volume, and such equilibria are more or less independent of temperature and pressure. In Type III (exemplified by myosin and methyl cellulose), however, the gelation reaction is endothermic and involves an increment (+ DV) in the volume of the system. Accordingly, such gels should be solated by increasing pressure (and decreasing temperature). The recent pressure measurements of Marsland and Brown (1942), on myosin and methyl cellulose have substantiated this relation (FIGURE 1).

The protoplasmic gels in the various cells which have been studied (and these include egg cells from several animal phyla, as well as various protozoa and plant cells) all fall into category III of the Freundlich classification. Regardless of the initial strength or stiffness of the gel structure in each different cell, increasing pressure induces a progressive weakening of the tensile properties of the gel which culminates in complete solation. Moreover, the solation curves are of a logarithmic nature, and, within the limits of the centrifugal method of determining the protoplasmic consistency, all the curves are very similar and approximately superimposable (FIGURE 1).

The Plasmagel System of Egg Cells. On the basis of recent observations, the egg cell may be compared to the *Amoeba*, in which the firmly gelated parts of the protoplasm are referred to as plasmagel, in contrast to the more

fluid regions, which are called the plasmasol. In the egg cell, the firmly set plasmagel includes the cortical layer of protoplasm immediately subjacent to the surface membranes as well as the spindle and the astral regions, whereas the deep-lying cytoplasm (aside from the asters and spindle) consists of plasmasol.

The existence of a cortical gel layer in the *Arbacia* egg and its possible importance in relation to cleavage was first indicated by the work of Brown

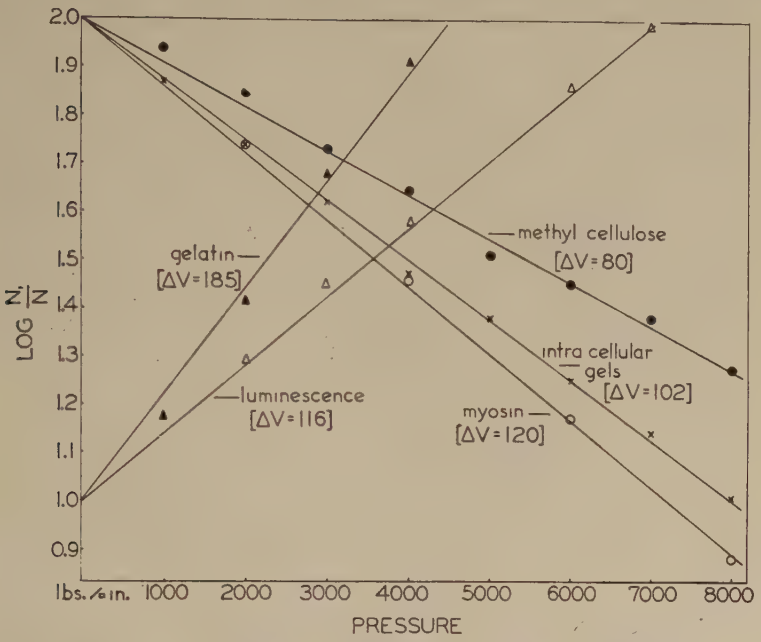

FIGURE 1. Effect of pressure upon various sol-gel equilibria. N_1/N equals the relative strength (or stiffness) of each gel, measured by the falling ball method (for gelatin, myosin, and methyl cellulose) or by centrifugation (various protoplasmic gels). In each case, the slope of the line evaluates ΔV, the cubic centimeter per mole change in volume which the system undergoes in passing from the sol to the gel state, or the reverse. Note that gelatin is a type I gel which stiffens as the pressure is raised (or as the temperature is lowered), whereas myosin and the other protoplasmic gels are type III, which undergo solation with increasing pressure (or with decreasing temperature). Myosin (from rabbit muscle): 2.1 per cent solution, 36.7° C., pH 6.72. Gelatin: 4 per cent solution, 22.0° C., pH 6.4. Intracellular gels: based on the average values obtained by measuring the plasmagel strength of *Amoeba* (2 species), *Arbacia* eggs (fertilized and unfertilized), and the leaf cells of *Elodea*.

(1934). Brown showed that the pigment granules in the cortex of the egg, compared to those in the deeper protoplasm, are more resistant to displacement in a weak centrifugal field. When the eggs were centrifuged under pressures ranging up to 8,000 lbs/in.,[2] however, the strength of the cortical gel was drastically reduced. This result, together with Costello's observation (1934) that the cortical gel of the *Arbacia* egg is weaker at low temperature, clearly indicated that the cortical gel of the *Arbacia* egg conforms with the Freundlich criteria for a Type III system. Moreover, there have been several other lines of evidence which demonstrate the gelated nature of the egg cortex. Motomura (1935) and Schechtman (1937) noted that the protoplasmic currents of the frog's egg, which appear at the time of the cleavage

furrow, involve only the deeper cytoplasm, and that visible granules in the peripheral gelled layer are not disturbed by the stream. Chambers (1938) determined that the thickness of the plasmagel layer in the furrow region of the *Arbacia* egg is about 5 microns, by the method of watching the furrow impinge upon a mass of oil which had been injected into the stalk between the blastomeres. Furthermore, Chambers has observed that, when one of the two potential blastomeres of a dividing egg is punctured, practically all of the internal cytoplasm pours out from the unpunctured blastomere, through the aperture surrounded by the impinging furrow, leaving a rind

FIGURE 2. A tenfold increase in the strength of the cortical gel of the egg-cell occurs just before the cell divides. All these cells were centrifuged simultaneously at the same high force (17,000 × gravity). In the two unfertilized eggs (at the left), the structure of the cortical gel is weak, allowing the pigment granules to be thrown into a black densely packed mass at the heavy end of the stretched cell, and completely clearing the hyaline zone. But in the one fertilized egg (at the right), five minutes before the furrow will appear, the pigment granules remain fixed in the strongly gelated cortex so that the cortex of the "hyaline zone" remains granular, a dense black pigment mass is *not* formed, and the cell remains unstretched by the high centrifugal force. *Arbacia pustulosa*, Naples Zoological Station.

of protoplasm which is especially thick bordering the furrow. This cortical remnant displays the properties of a gel, not only in that it does not participate in the flow of escaping plasmasol, but also in its behavior when manipulated with microneedles.

Formation of the Plasmagel Cleavage Girdle. After fertilization, just eight minutes before the first cleavage furrow will appear, the strength of the cortical gel increases to more than tenfold the unfertilized value. Now, a centrifugal force of 17,000 × gravity is unable to displace the cortical granules, whereas previously a force of 1,700 × gravity was more than adequate (FIGURE 2). Moreover, this newly fortified gel is particularly thick and strong in a bandlike region which engirdles the egg at the equator where the furrow is about to form (Marsland, 1939). Thus, it is reasonable to postulate that this gelated girdle exerts a contractile force which constricts the equator and effects the cleavage of the egg.

Retreat of the Furrow at High Pressures. When pressures above 5,000 lbs/in.^{2} are applied to the dividing egg, the progress of the furrow halts abruptly. Then the furrow recedes, slowly at lower, but quite rapidly at higher pressures (FIGURE 3). This effect is reversible, since refurrowing

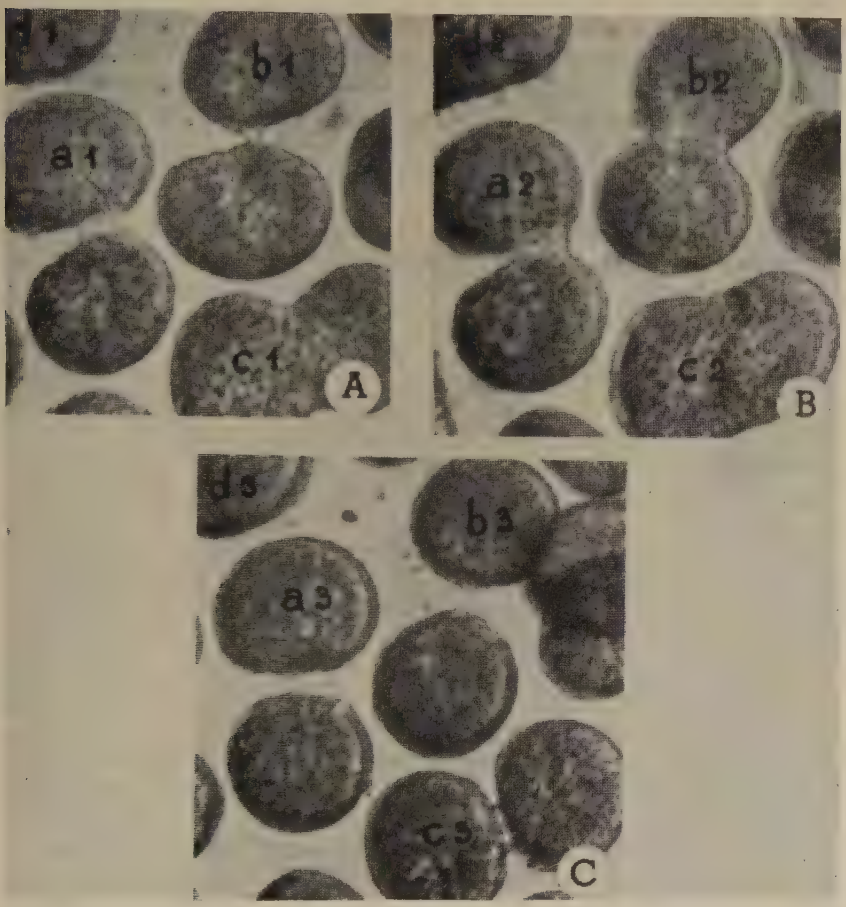

FIGURE 3. Retreat and advance of the cleavage furrow as the pressure is raised and lowered: A, eggs (*Arbacia punctulata*) 10 seconds after a pressure of 6,500 lbs./in.^{2} was established in the chamber; B, two minutes later, pressure still maintained; C, two minutes after decompression, which occurred immediately after photograph B was taken. Note (b_1 and b_2) that recession of the furrow occurred even though the "blastomeres" were connected by a mere strand of protoplasm at the time the pressure was applied. The fertilization membranes were removed shortly after the sperm was added.

commences as soon as the pressure is released. In fact, it is possible to induce the furrow to cut into the egg two or three times, by releasing the pressure after each compression period. If the high pressure is maintained for about 15 minutes, however, a furrow does not reappear immediately after decompression. On the other hand, when the time for second cleavage comes, a double furrowing usually occurs, which results in the production of three or four blastomeres. In many eggs, no apparent injury results from

a suppression of the first cleavage. They go through subsequent cleavages synchronously with control eggs and give rise to blastulae which cannot be distinguished from untreated specimens.

Retardation of the Furrow at Lower Pressures. Below 5,000 lbs/in.,^{2} pressure progressively retards the progress of the furrow from the equator to the spindle axis. In these experiments, the fertilized eggs were allowed to develop in the chamber at atmospheric pressure until four minutes before the furrows were expected to appear, at which time the pressure was raised quickly to the desired level. In order to time the progress of the intruding furrow accurately, it was found necessary to remove the fertilization membranes by shaking the eggs before placing them in the chamber. This procedure permits the dividing eggs to become more elongate in the direction of the division axis and enables one to see the furrows more plainly. In this way, it is possible to determine precisely when the furrow begins to form and when it finally reaches the axis of the dividing cell.

As the pressure is increased, there is a marked increase in the time required for the furrow to complete its passage from the equator to the axis. At 2,500 lbs/in.,^{2} the rate of progress of the furrow is only half the atmospheric rate, whereas, at 5,000 lbs., the progress is slowed to about one-fifth of the original rate. At pressures between 5,000 and 6,000 lbs., abortive furrows are formed which fail to reach the division axis before receding. Above 6,000 lbs., no furrows appear, although the eggs do become slightly elongate at the time when cleavage is due.

When one plots the rate of furrowing as a function of pressure (FIGURE 4), it is found that the retardation is in proportion to the solation effect which the pressure exerts upon protoplasmic gels generally. This would indicate an intimate relation between the mechanics of furrowing and the capacity of the plasmagel system to undergo a process of setting at the time when the furrowing is active. In other words, solation of the strongly gelled cleavage girdle is accompanied by a proportional reduction of its contractile strength. However, a direct measurement of the pressure solation of the exceptionally strong cleavage gel necessitated the use of relatively high centrifugal forces.

Solation of the Firm "Cleavage Gel." The eggs of *Arbacia pustulosa*, which are available at the Naples laboratory in abundant quantities, were chosen for these experiments. These eggs possess an unusually generous number of pigment granules, and this pigment is found, even in the unfertilized egg, predominantly in the cortical layer of the cytoplasm. These two attributes are of particular advantage in the experiments.

Previously, it had not been possible, using centrifugal forces up to 7,200 × gravity, and pressures up to 7,000 lbs/in.^{2} (Brown, 1934), to cause any appreciable displacement of the pigment granules if the centrifuging was done late in the division cycle, *i.e.*, within 10 minutes of the time when the furrows were due to appear. In the present experiments, it was found necessary to use a force of 16,500 × gravity to secure an adequately rapid displacement of the pigment granules through the firmly set plasmagel. But even this force is not sufficient to dislodge the granules, except under the liquefying action of pressures greater than 1,500 lbs/in.^{2} At atmos-

pheric pressure, a force of 18,680 × gravity, the highest pressure immediately available at the Naples laboratory, gave practically no pigment displacement, even with prolonged centrifugation.

Each sample of eggs, compressed to the desired degree, was centrifuged for a period just sufficient to displace the cortical pigment into a compact zone at the centrifugal pole of the cell. In each case, the eggs of a single female were fertilized and allowed to develop in the usual fashion until the

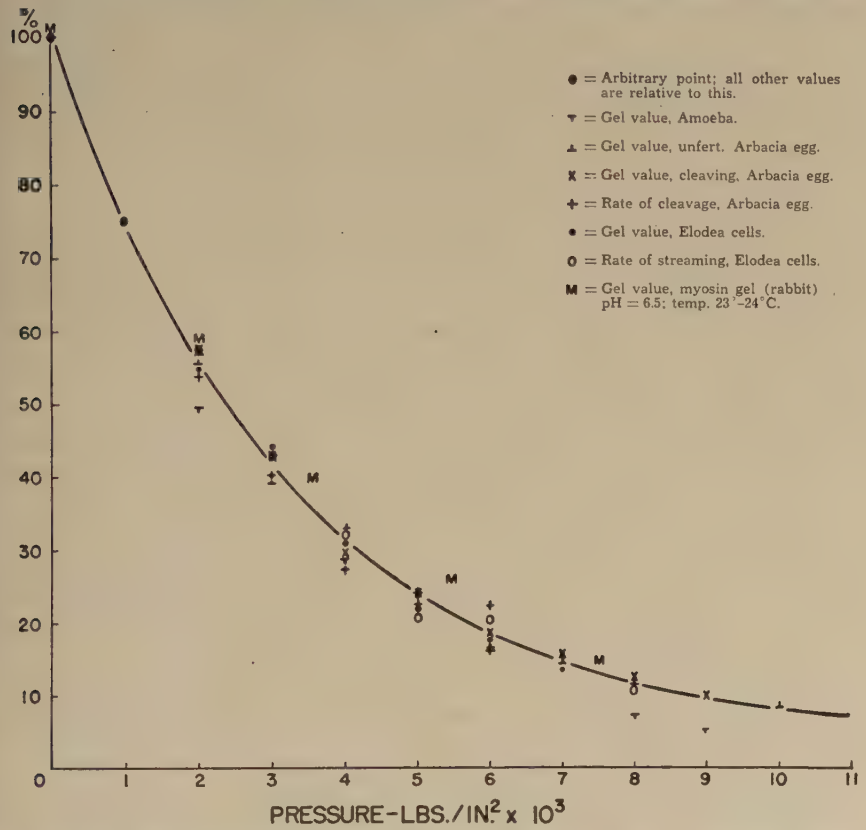

FIGURE 4. Measurements which indicate that protoplasmic contractility is lost in proportion to the decrease in plasmagel strength which occurs as the pressure is raised.

first furrows began to appear. Then, without delay, the control and experimental samples were placed in their respective sections of the centrifuge-pressure bomb, and the desired pressure was established in the pressure section. The operations were so fixed that, at the time when the pressure was applied and the centrifuging begun, about 50 per cent of the eggs possessed furrows of greater or lesser depth.

Assuming that the strength of the cortical gel is in proportion to the minimum centrifuge time required to produce a standard zoning of the pigment granules in the range of pressure wherein an effective displacement occurs, it is possible to plot the gelation values as a function of pressure. These

data (FIGURE 4) indicate, not only that the very firm plasmagel of the cleavage period shows the same relative susceptibility to the solating effects of pressure as other cellular gels, but also that the inhibition of furrowing is quantitatively related to the concomitant shifts in the sol-gel equilibrium.

The simplest and most direct explanation of the pressure effects upon the various egg-cells which have been studied is that cleavage results from the contraction of the equatorial girdle of gelated protoplasm. Just prior to the time of cleavage, the tensile strength of this gelated girdle undergoes a tenfold augmentation and the contractility of the gel appears to increase in proportion to this change. In the gelated state, the protoplasm apparently possesses contractile properties which are lacking in the solated system. Thus, the forces which deform the cell in both cleavage and amoeboid movement appear to be fundamentally similar in nature.

Effects of Pressure on the Spindle and Asters. That the spindle and asters of the dividing cell are also gel structures which are susceptible to dissolution under high pressure appears evident from the work of Pease (1941 and 1946) on the eggs of *Urechis* and the pollen mother cells of *Tradescantia*. No trace of the spindle or asters can be found in cells which are fixed immediately after a short exposure to pressures of 5,000–6,000 lbs/in.,[2] and these pressures completely abolish all anaphase movements of the chromosomes. When several minutes of recovery are allowed prior to fixation, however, the *Urechis* egg displays a number of cytasters which appear *de novo* and which possess peculiar "half-spindles" at points where contact is made with chromosomal material. Traction fibers are plainly visible in such "half-spindles," although interpolar fibers are absent, by the very nature of the configuration. Nevertheless, the "half-spindles" are capable of effecting a distinct displacement of the chromosomes toward the astral centers. Thus, it seems the experiments of Pease substantiate the view that "traction fibers" are gel structures which effect a translocation of the chromosomes by virtue of their contractility.

Conclusion. In summary, studies on the effects of pressure on the sol-gel equilibria of various cells indicate that gelation endows the protoplasm with contractile properties. In fact, these studies lead to the general conclusion that protoplasmic gelations are instrumental in the fulfillment of a variety of cellular movements, including cleavage, the translocation of the chromosomes during mitosis, amoeboid locomotion, and several other activities which have not been considered in this account.

Bibliography

BROWN, D. E. S. 1934. The pressure coefficient of "viscosity" in the eggs of *Arbacia punctulata*. J. Cell. & Comp. Physiol. **5:** 335.

CHAMBERS, R. 1938. Structural and kinetic aspects of cell division. J. Cell. & Comp. Physiol. **12:** 149.

COSTELLO, D. P. 1934. The effects of temperature on the viscosity of *Arbacia* egg protoplasm. Jour. Cell. and Comp. Physiol., **4:** 421.

FREUNDLICH, H. 1937. Some recent work on gels. J. Phys. Chem. **41:** 901.

MARSLAND, D. A. 1939. The mechanism of cell division. Hydrostatic pressure effects upon dividing egg cells. J. Cell. & Comp. Physiol. **13:** 15.

MARSLAND, D. A. 1942. Protoplasmic streaming in relation to gel structure in the cytoplasm. Chapter in "The Structure of Protoplasm." Monograph of the Society of Plant Physiologists, Ames, Iowa.

Marsland, D. A. & D. E. S. Brown. 1936. Amoeboid movement at high hydrostatic pressure. J. Cell. & Comp. Physiol. **8:** 167.

Marsland, D. A. & D. E. S. Brown. 1942. The effects of pressure on sol-gel equilibria, with special reference to myosin and other protoplasmic gels. J. Cell. & Comp. Physiol. **20:** 295.

Motomura, I. 1935. Determination of the embryonic axis in the eggs of Amphibia and Echinoderms. Sci. Reports of the Tohoku Imp. Univ. 4th series, **10:** 212.

Pease, D. C. 1941. Hydrostatic pressure effects upon the spindle figure and chromosome movement. I. Experiments on the first mitotic division of Urechis eggs. J. Morph. **69:** 405.

Pease, D. C. 1946. Hydrostatic pressure effects upon the spindle and chromosome movement. II. Experiments on the meiotic divisions of *Tradescantia* pollen mother cells. Biol. Bull. **91:** 145.

Schechtman, A. M. 1937. Localized cortical growth as the immediate cause of cell division. Science **85:** 222.

CLEAVAGE IN CENTRIFUGED EGGS, AND IN PARTHENOGENETIC MEROGONES

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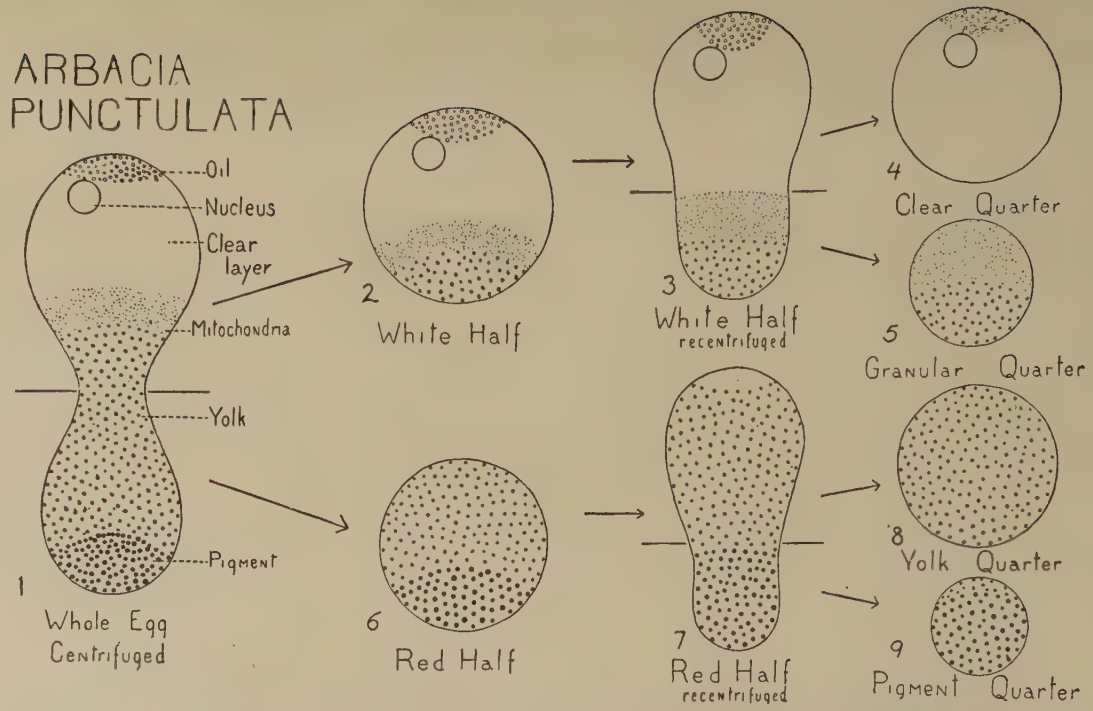
Centrifuged Arbacia punctulata *Eggs and Fractions.* When the *Arbacia punctulata* egg is centrifuged, the materials in the egg are segregated according to their density to form definite layers. These are: oil, at the light pole, clear layer, a narrow band of mitochondria, yolk, and pigment. The nucleus is always at the light pole under the oil (FIGURES 1, 10, 55). If centrifuged with a sufficiently high force, 10,000 × g, in a medium of the same density as themselves, so as not to be thrown to the bottom and crushed, the eggs elongate, become dumb-bell shaped, and then break into two "half-eggs" (FIGURES 2, 6), slightly unequal in size, but each half of a definite and constant size provided that a constant centrifugal force is used (E. B. Harvey 1932). The relative size of the two "half-eggs" varies with the amount of centrifugal force; the greater the force, the larger the heavy (red) half (Harvey 1941 a). The light (white) half contains the oil, the clear layer, all the mitochondria, and some of the yolk, with the nucleus under the oil. The heavy (red) half contains most of the yolk and all the pigment.

By further centrifuging at 10,000 × g, each of the "halves" elongates, becomes dumb-bell shaped (FIGURES 3, 7), and breaks into "quarter eggs" (FIGURES 4, 5, 8, 9) of a constant and quite definite size (Harvey 1932, 1936, 1940). The nucleus is always in the lightest (clear) quarter, which contains none of the readily visible granules. I specify "readily visible," meaning oil, mitochondria, yolk, and pigment. In well-centrifuged elongate white halves and clear quarters (Harvey 1946, Figures 30, 31), a thin band of very fine granules across the clear layer is to be seen on close observation. This divides the clear layer into two portions, differing in staining capacity with several vital dyes (Harvey 1941 c). The other three quarters are quite granular and contain no nucleus. In all these granular quarters there is, of course, some of the clear material or matrix in which the granules lie. This is shown particularly in the recentrifuged red half, where the further packing of the granules leaves a clear area at the centripetal pole (FIGURES 7, 59).

Polarity and Shape of the Centrifuged Arbacia *Egg. Arbacia* eggs do not orient in the centrifuge, so that the stratification has no relation to the original polarity. This was determined by Morgan and Lyon in 1907. There seems to be no doubt of this, since one can watch the eggs in the centrifuge microscope as they are thrown down, and there is no sign of orientation. The *Arbacia* egg differs in this respect from the *Chaetopterus* egg which, when centrifuged (in a sugar solution), does orient with the polar area toward the centripetal pole (Harvey 1939).

Arbacia eggs remain spherical if centrifuged for a short time with low forces, though they may be well stratified. They become elongate with



FIGURES 1–9. The unfertilized egg of *Arbacia punctulata*, stratified by centrifugal force (3 minutes at 10,000 × g), and the halves and quarters into which it breaks. The drawings are from camera lucida sketches and photographs, made as accurately as possible to scale. Magnified 400×. In FIGURE 9, the clear area at the centripetal pole is due to further packing of the granules with longer centrifugation. The actual sizes (diameters) are as follows: whole (spherical) egg 74 μ, white half 62 μ, red half 56 μ, clear quarter 56 μ, granular quarter 40 μ, yolk quarter 52 μ, and pigment quarter 32 μ.

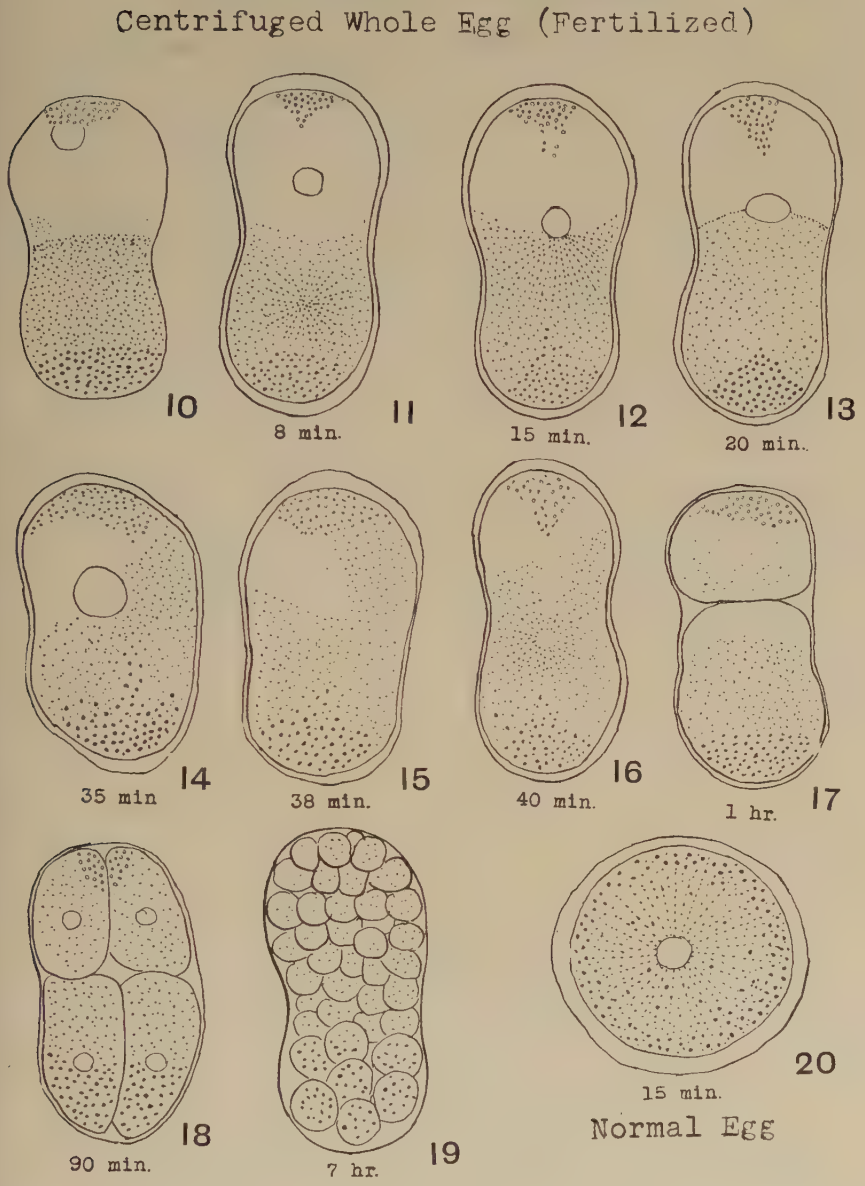
higher forces or with low forces for a longer period. After elongation, if not fertilized, they gradually resume their spherical shape and also lose their stratification. If fertilized immediately after centrifuging, they retain their elongate shape and the fertilization membrane follows the contour of the elongate egg.

Development of the Fertilized Centrifuged Egg and Fractions. The centrifuged whole egg, both spherical and elongate, and all its halves and quarters can be fertilized, form fertilization membranes, and cleave. Some of these will develop into normal plutei: the whole egg, the white half, the red half, and the clear quarter (Harvey 1932, 1936, 1940, 1946) (FIGURES 79–82).

In the spherical centrifuged whole egg, whether obtained by centrifuging with low forces, or by allowing the elongate egg to stand before fertilizing, the first cleavage plane usually comes in through the oil cap perpendicular to the stratification. This was observed in the pioneer work of Lyon reported in 1905, published in 1906. He used rather low forces, about 6,000 $\times$ g, and the eggs mostly remained spherical. He found (1907) that, in 90 per cent of the eggs, the first cleavage was perpendicular to the plane of stratification, the furrow always starting at the oil cap. In a few eggs, the cleavage came in equatorially, and in a few, at an angle. I can confirm this early work of Lyon. The second cleavage plane usually comes in equatorially, dividing the egg into two colorless and two pigmented cells, though it may come in through the poles. Normal blastulae, gastrulae, and plutei are formed, and the segregation of pigment remains through the early pluteus. It may be in any position in the pluteus, but most commonly in the mouth region (FIGURES 77, 78) as noted by Lyon (1907).

In the elongate centrifuged egg, when fertilized, the fertilization membrane, as mentioned above, follows the contour of the egg. Although there is usually a slight contraction away from the centripetal pole immediately following fertilization, leaving a larger perivitelline space here, the egg retains its elongate shape throughout cleavage and gives rise to a slipper-shaped blastula. The first cleavage plane almost always comes across the short axis of the egg, parallel with the stratification, dividing the egg into a smaller clear cell and a larger granular cell (FIGURES 17, 56). This is due to the position of the spindle in the long axis of the egg, where there is space for it. The second cleavage usually comes in perpendicular to the first in the clear cell and either perpendicular to, or parallel with, the first in the granular cell (FIGURES 18, 57). The second and following cleavages are asynchronous, the smaller clear cells dividing more rapidly than the larger pigmented cells. A slipper-shaped blastula is formed (FIGURES 19, 58). The pigmented area remains distinct throughout the cleavages and in the early pluteus, as in the spherical egg described above.

The white half, when spherical, cleaves quite regularly (FIGURES 28–32). The first cleavage plane divides the egg into two equal cells and may be in any position with regard to the oil cap (FIGURES 28–30). In the elongate white half, the spindle forms in the long axis and the first cleavage plane is parallel with the stratification, dividing the egg into a smaller clear cell and a larger granular cell, just as in the elongate whole egg (see Harvey 1940,

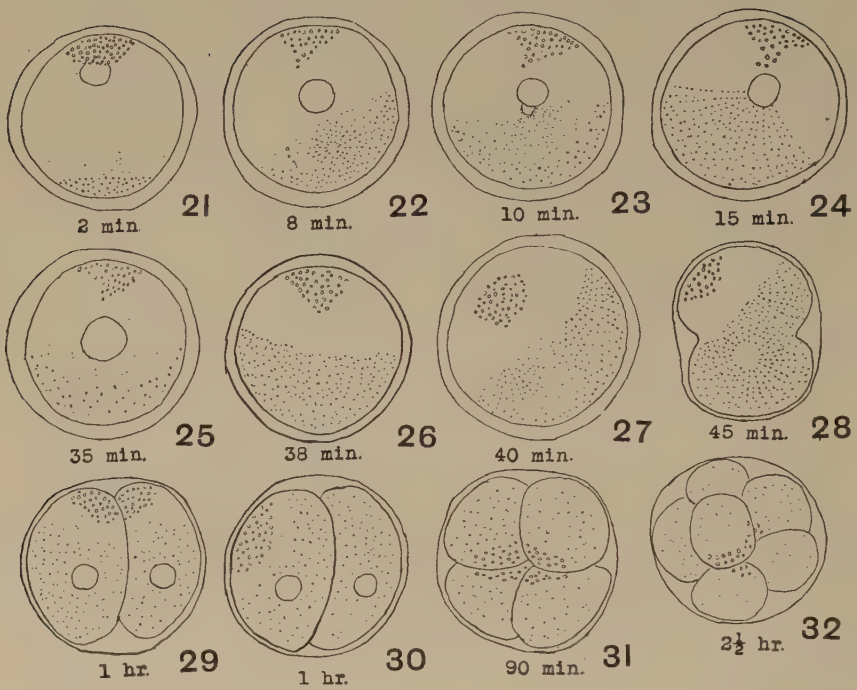


FIGURES 10–20.

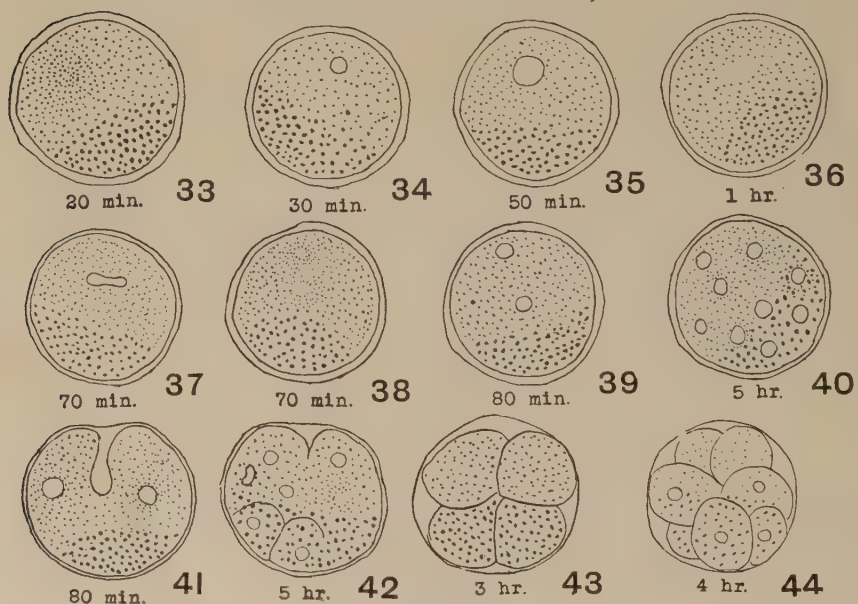
Figure 34). Perfectly normal but small white plutei develop, which in two or three days acquire the red pigment spots characteristic of the normal pluteus (FIGURE 80).

The red half, when fertilized, has only the male nucleus; that is, it is a fertilized merogone. The cleavages are sometimes quite regular (FIGURES

White Half (Fertilized)

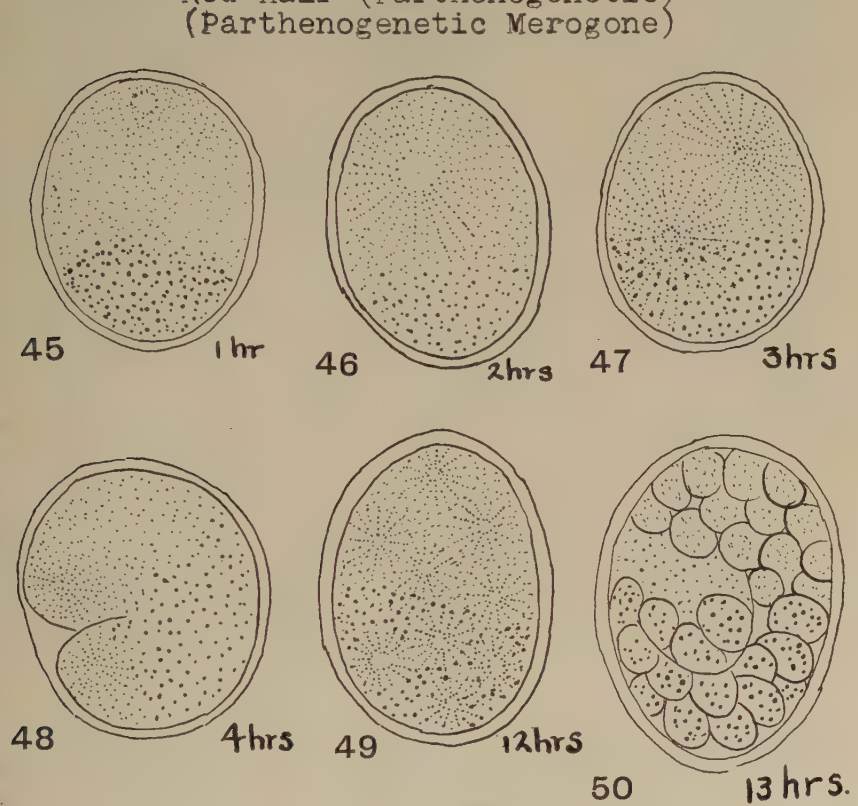


Red Half (Fertilized)



FIGURES 21–44.

43, 44), but in many cases the furrows fail to come in after nuclear division, even the first cleavage plane (FIGURES 39–42). Normal multicellular blastulae may be formed, and some perfectly normal plutei have been raised. These are small and very heavily pigmented (FIGURE 81). Development is much slower than in the whole eggs and white halves. In general, this is true of all eggs with only one nucleus, whether parthenogenetic or merogonic.



FIGURES 45–50.

The clear quarter, obtained by recentrifuging the white half, has none of the mitochondria, yolk, or pigment and is about as near to pure living protoplasm as it is possible to get. Nevertheless, it may develop quite normally (Harvey 1946), much like the white half. The first cleavage divides the cell equally and may be in any relation to the oil cap. It is greatly delayed, taking place in two hours instead of 50 minutes. The second cleavage is perpendicular to the first, and subsequent cleavages occur as in the normal egg and white half, though they are all greatly delayed. Though many of the clear quarters do not develop, I have obtained several plutei, quite normal except that they are small and clear (FIGURE 82). Later, they ac-

FIGURES 51–62. (*See opposite page.*)

quire the red pigment granules characteristic of the pluteus from a normal whole egg.

Parthenogenesis and Parthenogenetic Merogony. The whole centrifuged egg and the white half may develop parthenogenetically just like the normal egg, except that all stages are delayed. Normal plutei may be formed. We have seen that the red half may develop when fertilized. This has only the male nucleus and is therefore a fertilized merogone. By treating the red half with a parthenogenetic agent instead of fertilizing it, it has been found possible to obtain cleavage and some development without any nucleus at all. This I have termed "parthenogenetic merogony." Cleavage and early development take place as in the fertilized merogone, but more slowly in all stages (Harvey 1936, 1940). In the elongate eggs, the first cleavage comes across the short axis, but usually not in the narrowest part (FIGURE 60). Cleavages may take place in a fairly orderly fashion and we may get a many-celled blastula (FIGURES 50, 61, 62, AND 73). In many cases, however, cleavage planes fail to come in, as in the fertilized merogone, and the egg may be filled with many asters with clear centers, giving somewhat the appearance of craters on the moon (FIGURES 49, 75). Or the egg may be peppered with small clear spheres without radiations, giving the appearance of nuclei (FIGURE 76). These blastulae, both cellular and noncellular, have been observed while emerging from the fertilization membrane; the cellular ones usually break up at this time, but the noncellular ones have lived for a month, but without further development. Unfertilized eggs, at room temperature, live only a day or two. I have never been able to assure myself that they acquire cilia or become free-swimming. If they have cilia, they are short and irregularly spaced, and, if they move, it is only very slightly. The older parthenogenetic merogones do not increase in size. In fact, they become smaller and gradually lose their pigment. They are merely small spherical masses of more or less undifferentiated protoplasm. I am inclined to believe that, without a nucleus, multiplication of cells takes place, but not differentiation.

In a plate of photographs (FIGURES 51–62), one may see the similarity in development of the whole normal egg, the centrifuged egg, and the parthenogenetic merogone, *i.e.*, the egg without any nucleus whatever.

Cytological Details. In the fertilized centrifuged whole egg (FIGURES 11–16), one can see the sperm aster approaching the female nucleus which has descended from its position under the oil. In the *Arbacia* egg, both normal and centrifuged, the sperm may enter at any point on the surface. Here it has entered near the heavy pole. Then the two nuclei meet and we have the monaster stage (FIGURE 12). Where granules are lacking, the rays cannot be seen in the living egg, as rays are always indicated by lines of granules. Compare the monaster stage of an uncentrifuged egg (FIGURE 20). The oil cap breaks up, and oil droplets move downward while the pigment granules move upward. This is an indication of currents present in the protoplasm of the fertilized egg. The nucleus enlarges (FIGURES 13,

FIGURES 51–62 (*see facing page*). Photomicrographs showing comparative stages in the development of the normal uncentrifuged egg, the centrifuged egg, and the parthenogenetic non-nucleate red half. Living. Magnification 250×.

14) and the nuclear membrane breaks (FIGURE 15), just as in the normal egg. A typical amphiaster is formed, the rays in the clear portion again not being visible (FIGURE 16). The spindle cannot be seen in the living egg, normal or centrifuged. Cleavage takes place, as described above, typically dividing the egg into a smaller clear cell and a larger granular cell (Figures 17, 56).

The details of the fertilized white half (FIGURES 21–28) are the same and quite like the normal egg, except that the rays of the monaster and amphiaster cannot be seen where granules are lacking.

In the fertilized red half, which has no female nucleus, the sperm aster can be readily seen in the living egg (FIGURE 33). The male nucleus enlarges and breaks and a typical amphiaster is formed (FIGURES 34–38). In some cases, a dumb bell-shaped nucleus is observed, as though the nucleus pinched in two (FIGURE 37). As stated above, cleavage planes often fail to come in and we get a multinucleate egg (FIGURES 39, 40), but often the cleavages are normal and equal.

In the parthenogenetic merogone, the red half or yolk quarter, artificially activated and thus having no nucleus whatever, there is often to be observed, about an hour after activation, a clear area near the light pole, often spherical (FIGURES 45, 74). This must come from the clear material at the light pole, which segregates out in the centrifuging of the unfertilized red half (FIGURES 7, 59). One might call this a pseudonucleus, since it obviously contains none of the chromatin material of the original egg nucleus. Whether it has a membrane around it, I cannot say, but there is certainly, in many cases, a phase boundary separating it from the surrounding granules.

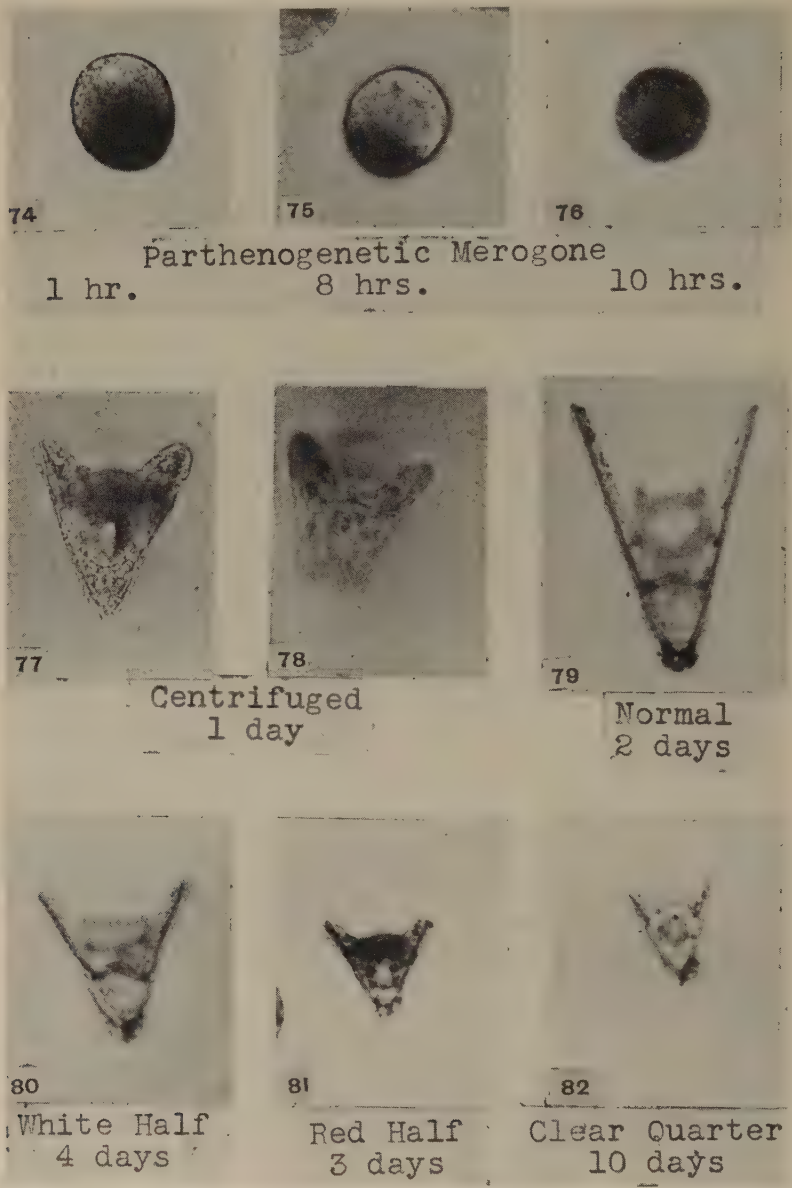
Around this pseudonucleus, astral rays develop and we have a typical monaster stage (FIGURE 46). Then a typical amphiaster is formed (FIGURE 47), and cleavage comes in between the two asters, beginning at the side of the egg nearest the amphiaster (FIGURE 48), as it does in normal fertilized eggs. It seems extraordinary that a monaster should arise first, preliminary to the amphiaster, when there is no sperm, with its accompanying centrosome, or a female nucleus which might supply a centrosome. Of course, asters do arise in the cytoplasm, usually many of them at once, after various treatments, *e.g.*, hypertonic sea water, various salts, and strychnine. Wilson (1901) has described them as capable of division. In the parthenogenetic merogones, however, there is a perfectly orderly sequence of events: a large monaster arising *de novo* without any pre-existing centrosome, then an amphiaster, then cleavage. The later development of the parthenogenetic merogone has been described above.

In fixed and stained sections of the parthenogenetic merogones, the asters are seen to be well formed and to occur in pairs, but there is no spindle, and there are no chromosomes. A comparison of three stages of a normal fertilized whole egg, a fertilized merogone, and a parthenogenetic merogone is given in FIGURES 63–71, showing the difference in the mitotic figure. In comparison with the whole egg, the spindle of the fertilized merogone is much more slender, and the rays of the aster are short and thick, especially

FIGURES 63–71. Sections of comparative stages of the division figure of a normal whole egg, a fertilized red half, and a parthenogenetic merogone. Stained with iron hematoxylin.

FIGURES 72 and 73. Aceto-carmine preparations of a fertilized merogone and a parthenogenetic merogone. Note the absence of chromatin-staining material in the latter.

in the pigment area. This is true also of the asters in the parthenogenetic merogone, both the monaster as shown in FIGURE 69, and the amphiasters as shown in FIGURES 70 and 71. The last photograph (71) shows a par-



FIGURES 74–82. FIGURE 74. A parthenogenetic non-nucleate red half, one hour after activation, showing the nuclear like body. FIGURE 75. A parthenogenetic red half, 8 hours after activation, showing many asters FIGURE 76. A little later stage of the parthenogenetic merogone, with many small vesicles resembling nuclei. FIGURES 74–76. Photomicrographs of living eggs. Magnification 250×. FIGURES 77 and 78. Young plutei from fertilized centrifuged eggs, showing the localization of pigment still persisting around the gut in FIGURE 77 and in one arm in FIGURE 78. FIGURES 79–82. Plutei from the whole egg, white half, red half, and clear quarter. FIGURES 77–82. Photomicrographs of living plutei (chlorotoned). Magnification 125×.

thenogenetic merogone going into a two-cell stage, and beside it about an 8-cell stage, the asters in pairs. There is no spindle and there are no chromo-

somes. The Feulgen reaction is negative, showing that there is no chromatin present.

Aceto-carmine preparations of the whole blastula of a fertilized merogone and a parthenogenetic merogone (FIGURES 72, 73) show very clearly the presence of mitotic figures and red staining chromosomes in the former, and their absence in the latter. As noted before, there are many small vesicles in the latter but they take no stain.

Conclusions

From the above account, we see that no special granules are necessary for cleavage and development (oil, yolk, pigment, and mitochondria), since any fraction may develop without one or more of these, and the clear quarter, which lacks all of them (except oil), may develop into a normal pluteus. As for the nuclei, it is apparent that the male nucleus is not necessary for cleavage and development, since parthenogenetic development takes place in many forms, and, in *Arbacia*, results in a perfect pluteus in both the whole eggs and the white halves. The female nucleus is not necessary, since merogonic development of fertilized non-nucleate fragments takes place in many forms and may result in a perfect pluteus in the red halves of *Arbacia*.

It has been shown that cleavage and early development may take place, in the parthenogenetic merogones, without any nucleus at all. The essential material for cleavage and early development, therefore, must be the matrix or clear material which is present in all the fractions. This granule-free protoplasm, which is, according to E. N. Harvey (1932), 61.1 per cent of the egg, has a density (*ca.* 1.04) and a viscosity (*ca.* 3 centipoises) not greatly different from that of sea water, according to Heilbrunn (1926, 1943, p. 69). In the living state, it is optically empty, except for the thin line of granules segregated out under high centrifugal forces, as mentioned before. When fixed and stained with haemotoxylin, however, it appears filled with very small granules (Lyon 1907, Harvey 1940, Figure 124). It contains nucleo-proteins, as shown by ultraviolet of a wave length of 2537 Å (Harvey and Lavin 1944). It contains the greater part of the enzyme peptidase, as determined by Holter (1936). It can be separated by centrifugal force, as mentioned above, into a lighter portion and a heavier portion, which react differently to various vital dyes (Harvey 1941 c). The ordinary granules must have some importance and may be used even in early development, but they are not essential and can be replaced. At least one nucleus seems to be necessary for complete development, but the early stages of development involving cell multiplication can take place without any nucleus.

Bibliography

HARVEY, E. B. 1932. The development of half and quarter eggs of *Arbacia punctulata* and of strongly centrifuged whole eggs. Biol. Bull. **62:** 155–167.

HARVEY, E. B. 1936. Parthenogenetic merogony or cleavage without nuclei in *Arbacia punctulata*. Biol. Bull. **71:** 101–121.

HARVEY, E. B. 1939. Development of half-eggs of *Chaetopterus pergamentaceus* with special reference to parthenogenetic merogony. Biol. Bull. **76:** 384–404.

HARVEY, E. B. 1940. A comparison of the development of nucleate and non-nucleate eggs of *Arbacia punctulata*. Biol. Bull. **79:** 166–187.

Harvey, E. B. 1941a. Relation of the size of "halves" of the *Arbacia punctulata* egg to centrifugal force. Biol. Bull. **80:** 354–362.

Harvey, E. B. 1941c. Vital staining of the centrifuged *Arbacia punctulata* egg. Biol. Bull. **81:** 114–118.

Harvey, E. B. 1946. Structure and development of the clear quarter of the *Arbacia punctulata* egg. J. Exp. Zool. **102:** 253–276.

Harvey, E. B. & G. I. Lavin. 1944. The chromatin in the living *Arbacia punctulata* egg and the cytoplasm of the centrifuged egg as photographed by ultra-violet light. Biol. Bull. **86:** 163–168.

Harvey, E. N. 1932. Physical and chemical constants of the egg of the sea urchin, *Arbacia punctulata*. Biol. Bull. **62:** 141–154.

Heilbrunn, L. V. 1926. The absolute viscosity of protoplasm. J. Exp. Zool. **44:** 255–278.

Heilbrunn, L. V. 1943. An Outline of General Physiology. Second edition. Saunders Co. Philadelphia.

Holter, H. 1936. Studies on enzymatic histochemistry. XVIII. Localization of peptidase in marine ova. J. Cell. & Comp. Physiol. **8:** 179–200.

Lyon, E. P. 1906. Some results of centrifugalizing the eggs of *Arbacia*. Amer. J. Physiol. **15:** xxi–xxii (follows p. 301).

Lyon, E. P. 1907. Results of centrifugalizing eggs. Arch. Entw.-Mech. **23:** 151–173.

Morgan, T. H. & E. P. Lyon. 1907. The relation of substances of the egg, separated by a strong centrifugal force, to the location of the embryo. Arch. Entw.-Mech. **24:** 147–159.

Wilson, E. B. 1901. Experimental studies in cytology. I. A cytological study of artificial parthenogenesis in sea urchin eggs. Arch. Entw.-Mech. **12:** 529–596.

THE EFFECTS OF ULTRACENTRIFUGAL FORCE ON THE CELL WITH SPECIAL REFERENCE TO DIVISION

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Relatively few protoplasmic components and inclusions are markedly affected by gravitational force. However, a few cellular materials differ sufficiently in density to be displaced through the cell by gravity. Examples of such materials are the yolk of the frog's egg (Schultze, 1894), nucleoli of *Echinus* (Gray, 1931), and possibly certain large plastids of plant cells. Smaller components and inclusions, which differ only slightly in density from those of the surrounding protoplasm, are relatively unaffected by gravity. This is true, not because these constitutents are all of the same relative density as the cytoplasm, but because the structure, consistency, and particularly the diffusion within the protoplasm oppose their partial or complete sedimentation. Some of these materials that are unaffected may be readily displaced by relatively low centrifugal force without seriously interfering with normal development. This is especially true if the stratified materials undergo a redistribution before the first cell division. Furthermore, the *Arbacia* egg may even be divided into as many as four separate components, each of which contains cellular constituents of different relative densities, yet each component is capable of subsequent development (E. B. Harvey, 1932, 1933).

On the other hand, mosaic eggs (*e.g.*, the Ascidian, Conklin, 1931), in addition to showing a stratification of the usual ooplasmic substances, often have the formative or organ-forming materials displaced, giving rise to various types of abnormal development. Costello (1939) has determined the volumes occupied by the formed cytoplasmic components in certain marine eggs.

Types of Ultracentrifuges. Among the earliest experiments on the effects of centrifugal force upon the biological material was that of Knight (1806). He devised a water wheel by which he was able to show that centrifugal force could overcome the pull of gravity in directing the growth of seedlings. Since that time, many types of centrifuges have been devised, starting with the relatively simple hand centrifuges; electrically driven centrifuges; Sharples super centrifuges; oil turbine ultracentrifuges of Svedberg and associates (see Svedberg and Pedersen, 1940, for literature); air turbine ultracentrifuges, first described by Henriot and Hugunard (1925, 1927) and later developed into a practical laboratory apparatus by J. W. Beams and associates (1930, 1931, 1933); air turbine-vacuum type ultracentrifuges (J. W. Beams, 1937, 1938 for literature); and, finally, the recently developed magnetically supported and magnetically driven vacuum type ultracentrifuge (J. W. Beams, 1947). This last machine, while still in an experimental stage of development, has been able to spin a very small steel ball with a diameter of .521 mm. at 633,000 R.P.S., thus producing an acceleration of 1.04×10^8 gravity before bursting.

In general, it may be said that one of the most important advances in centrifuge techniques, in addition to the development of high rotational speeds, is the spinning of the rotor in an atmosphere of hydrogen (Svedberg) or partial vacuum (J. W. Beams). Such a procedure, particularly when the centrifuge is operated at high speeds, greatly reduces the temperature gradient, which is the main cause of convection and hence of the remixing of the solution.

In addition to the above-mentioned centrifuges with high rotational speeds, Harvey and Loomis (1930) devised an optical system (centrifuge-microscope) which gives an excellent image of living cells while they are rotating. This same principle has been adapted by E. N. Harvey (1932, 1934) to the air turbine rotor, with the result that a good image of living cells may be obtained while centrifuging at 200,000 times gravity. Pickles (1936) has also described an optical scheme suitable for observing rotating cells at high speeds. (For the literature concerning sedimentation in high centrifugal fields, the reader is referred to the works of Svedberg and Pedersen (1940) and J. W. Beams (1947).)

Recently, E. N. Harvey (1946) has calculated the density gradients necessary to separate certain X- and Y-bearing spermatozoa on the basis of their relative size and density. He suggests that the separation of the spermatozoa on this basis is theoretically possible and could probably be experimentally achieved in the air turbine-vacuum type ultracentrifuge.

It is beyond the scope of this paper to review the extensive literature related to the use of the ultracentrifuge in connection with the determination of molecular weights, sizes, and shapes of both naturally occurring and synthetic solutions. (References to this literature may be found in the works of Wyckoff, 1937a, 1937b; Svedberg and Pedersen, 1940; Stanley, 1939; Stern, 1943; and others.)

Visible Cell Constituents. The chief use of the ultracentrifuge in cytology has thus far been on cells of relatively small size and with comparatively high viscosity, where the ordinary laboratory centrifuge produces little effect.

(*a*) *Golgi Apparatus.* One of the first experiments made on tissue cells by the ultracentrifuge method was the displacement of the Golgi apparatus to the centripetal ends of the uterine gland cells of the guinea pig (Beams and King, 1934a). Thus, it was concluded that the Golgi apparatus is lighter than the surrounding cytoplasm and is probably composed in part of lipoid material. Similar studies on the Golgi apparatus in other types of cells have been made by Guyer and Claus (1936a, 1936b), Beams, Gatenby, and Muliyil (1936), Dornfeld (1936, 1939), Beams and King (1938), Singh (1938), Daniels (1938), Beams and Sheehan (1941), Bourne (1942), and others. The importance of these experiments has been to supplement the existing data confirming the fact that the Golgi apparatus is a real cellular constituent, whose function must be considered in any attempt at a complete analysis of the physiology of the cell as a whole. Perhaps a chemical analysis of the Golgi apparatus like that made for mitochondria (Bensley, 1937) may be accomplished soon.

The Golgi apparatus has been observed in living cells (Gatenby and

Moussa, 1949; and others). Nevertheless it has been called a "myelin figure, or a complex of myelin figures, artificially induced in cells during preparation" (Palade and Claude, 1949).

The osmiophilic platelets of plant cells thought to be homologous to the Golgi apparatus of animal cells by Bowen (1928) are likewise readily displaced to the centripetal end of the cell (Beams and King, 1935b).

(b) *Mitochondria.* Mitochondria, too, have been stratified in the cell by high centrifugal force. Thus, their relative density is usually greater than that of the surrounding cytoplasm but often not so dense as certain other cellular inclusions. Such experiments demonstrate that these bodies are discrete structures. However, their function is still debatable (Brown, 1914; Conklin, 1931; Harvey, 1932, 1933; Beams and King, 1934b; Beams, Gatenby, and Muliyil, 1936; Patten and Beams, 1936; Bourne, 1942; Claude, 1943; Monné, 1944; Clement, 1938; Holter and Doyle, 1938; and others). It has recently been shown that all the principal oxidative enzymes of the animal cell are associated with the mitochondria (refer to Green, 1949).

In the germ cells of *Helix*, the Golgi apparatus may be observed at the centripetal end, while the mitochondria collect at the centrifugal end of the same centrifuged cell. Here a sharp demarcation between these two cellular elements occurs—an argument against the thought that the Golgi apparatus is only a form of mitochrondria (Beams, Gatenby, and Muliyil, 1936).

Mitochondria have recently been separated and concentrated by differential centrifugation, thus permitting accurate chemical analysis (Bensley and Hoerr, 1934; Hogeboom, Schneider, and Palade, 1948). Bensley (1937) published the following approximate composition for dried-liver mitochondria: proteins and unknowns 64.67 per cent; glycerides 28.88 per cent; lecithin and cephalin 4.2 per cent; and cholesterol 2.25 per cent. The work of Harvey (1932, 1936) demonstrates that certain half or quarter eggs of *Arbacia* may undergo development in the apparent absence of mitochondria.

(c) *Nissl Bodies and Neurofibrillae.* The state in which the Nissl substance exists in the nerve cells is a question of long standing and of currently conflicting opinion. Since the Nissl bodies cannot be seen in the living cell, they are said by some to be artifacts, appearing only in fixed and stained preparations as a result of coagulation and precipitation of a dissolved material spread uniformly throughout the nerve-cell cytoplasm. A centrifugal force of 400,000 times gravity for 30 minutes displaces the Nissl bodies to the centrifugal end of the cell (Beams and King, 1935). After being moved through the cytoplasm, they retain their discreteness. This argues for the view that the Nissl substance is not uniformly distributed through the nerve cell but is aggregated in composite masses as typically observed in histological preparations.

Neurofibrillae, too, may be greatly distorted and actually displaced by high centrifugal force. Here again, the centrifuge method has furnished additional evidence for the presence of fibrillae in certain neurons, the presence of which has been denied by some (Beams and Kirshenblit, 1940). In addition, the ultracentrifuge method indicates that the spinal ganglion cells of the rat are highly viscous.

(d) *Plastids.* Many workers have observed that relatively low centrifugal

force is sufficient to displace plastids of various kinds. Thus, they are usually much denser than the surrounding cytoplasm. (See Beams and King, 1939, for a review of this subject.) In addition, the flowing cytoplasm of *Elodea* may be displaced centrifugally, consequently disrupting cyclosis. Upon removal from the centrifuge, however, the displaced cytoplasm is rapidly churning and eventually reestablishes its flowing movements. Considerable time is required, however, for a complete breakup of the clumped chloroplasts, which have a tendency to stick together when massed. The large clumps of chloroplasts cause considerable damming of the newly established streams of protoplasm. The nucleus, too, in such cells is often free and has been observed to circulate in the streams of the flowing protoplasm.

(e) *Secretion Products.* Secretion products such as yolk, zymogen granules of the pancreas, salivary glands, thyroid secretion, secretion granules of the liver (Claude, 1943), and granules of leucocytes have all been observed to be affected by high centrifugal force.

(f) *Vitamin C Granules.* Bourne (1942) found that the granules which he interprets as containing vitamin C were displaced by the ultracentrifuge. They seem to take up the same position as the Golgi apparatus in some cells, while in others they were stratified with the mitochondria. Increased evidence seems to indicate that vitamin C is in some way associated with the Golgi apparatus. However, very little information is available concerning its distribution in eggs and embryonic cells. This subject should provide a fertile field for further research.

(g) *Stratification of the Mammalian Erythrocyte.* Considerable controversy exists concerning the internal architecture of the mammalian erythrocyte (Ponder, 1934). When ultracentrifuged at 400,000 times gravity for 30 minutes and subsequently fixed and stained, two, and sometimes three, definite layers may be observed. The layers have not thus far been analyzed. However, they may be differentially stained and in some cells the hemoglobin seems to be thrown centrifugally (Beams and Hines, 1944; Beams, 1947). Erythrocytes of certain Amphibia may be greatly stretched and pulled into nucleated and non-nucleated halves without cytolysis (Beams and King, 1945). The various white blood cells may likewise be stratified by high centrifugal force.

(h) *Particulate Bodies of Cytoplasm.* Certain small particulate bodies, ranging from just visible to submicroscopic size, have been isolated from the cytoplasm by Claude (1940), Bensley (1943), and others. These bodies have been stratified in the *Amphiuma* liver by ultracentrifugal force (Claude, 1943). According to Claude, these small particles seem to represent well-defined morphological constituents of the ground substance of the cell. (For further discussion concerning these bodies see Bensley, 1943; Lazarow, 1943; Hoerr, 1943; and Claude, 1943.)

(i) *Nucleus and Nuclear Components.* Many of the early works on centrifuging described some displacement of the nucleus as a whole, as well as a stratification of its component parts. However, high centrifugal force produces a more marked displacement of the nucleus through the cytoplasm and a more striking stratification of its component parts. Usually, the

nucleus stratifies from the centrifugal to the centripetal end as follows: nucleolus, chromatin, linin, karyolymph. Often, the nucleolus and chromatin cause the nucleus to be greatly stretched, and in some cases they actually rupture it and come to rest free in the cytoplasm. In *Paramecium*, the chromatic portion of the macronucleus may be separated from the achromatic portion. Other references to the effects of centrifuging on Protozoa may be found in the paper by Beams and King (1941). Certain bacteria also appear to have their intracellular granules displaced by ultracentrifugation. Some of the displaced granules probably represent chromatin material (King and Beams, 1942).

Even the nucleolus in *Nereis* eggs may be stratified into a centrifugal granular and centripetal clear region (Costello, 1940).

(j) *Intracellular Virus Bodies.* In addition to the concentration of virus material removed from cells (*cf.* Stanley, 1939; Stern, 1943, for literature), the ultracentrifuge method has been used to study intracellular virus bodies of living cells. Lucas and Herrmann (1935) have concluded that the herpetic intranuclear inclusions of the rabbit cornea are lighter than either the nuclear sap or the chromatin. On the other hand, similar inclusions in the salivary glands of the guinea pig are heavier than the nuclear sap (Rosenbush and Lucas, 1939). Here, either the densities of the virus bodies vary in different types of cells or, more likely, the relative density of the protoplasm differs, either of which would account for the above results.

(k) *Chromosome Structure.* As pointed out by Kostoff (1937, 1938), if the chromosome is a heterogeneous body, it should be possible to break the chromosomes by centrifuging and displace their component parts. He observed that, in addition to the production of the chromosome fragments, chimeras, and many types of genetic abnormalities, certain anaphase chromosomes which were destined for one pole were displaced to the opposite one, resulting in monosomic, trisomic, polysomic, and tetrahaploid cells.

The effects of high centrifugal fields on the structure of the grasshopper chromosomes indicate that, in addition to producing an extensive stretching, ultracentrifuging displaces some of the component parts. Some of the chromosomes have been interpreted as showing a displacement of a hematoxylin-staining material which probably represents a portion of either the chromonemata or matrix (Beams, 1948).

Preliminary unpublished observations indicate that this method may be of value in connection with the study of salivary gland chromosome structure.

Displacement of Certain Surface or Membrane Materials. C. V. Taylor (1931) noted that, during the subsequent development of the centrifuged immature eggs of *Urechis caupo*, the fertilization membrane first appears, and is thicker, at the centrifugal end. E. B. Harvey (1932) observed a thicker fertilization membrane on the centrifugal half of eggs of *Arbacia* than was present on the centripetal halves. Similar observations were reported by Costello (1935) for *Asterias*. In addition, the ectoplasmic or hyaline plasma layer of some sea urchin eggs may be completely removed by centrifuging (Harvey, 1934).

Costello (1940) reports that ultracentrifuged *Nereis* eggs often have most

of the cortical jelly-precursor of the vitelline membrane displaced to the centrifugal end of the egg.

These results are interesting in view of the theories which hold that the cortical substances of eggs are not displaced by centrifugal force.

Tension at the Surface of Living Cells. E. N. Harvey and associates have used the centrifuge method (both high and relatively low centrifugal force) to measure the tension at the surface of various cells which do not possess protective or gelled surface layers. They allowed the cells to ingulf oil droplets or they injected oil droplets directly into them and measured the force necessary to pull the droplets through the cell membrane. Values for the tension at the surface in *Arbacia* eggs were 0.2 dyne/cm. (E. N. Harvey, 1931); for *Amoeba dubia* 1 to 3 dynes/cm. (Harvey and Marsland, 1932); and rabbit macrophages 2 dynes/cm. (Shapiro and Harvey, 1936). For values of the tension at the surface of other cells, see E. N. Harvey (1938).

Viscosity Studies. Viscosity measurements on living cells may be made by several different methods. However, much of the information concerning the absolute and relative viscosity of marine eggs has been gained by use of the centrifuge method, using relatively low force. (For results, literature, and techniques involved in this method, see Heilbrunn, 1921, 1928.)

By aid of the ultracentrifuge, Guyer and Claus (1939, 1942) have extended this method to the study of the relative viscosity of several different types of tumor cells. In general, they found that, when normal as well as tumorous cells from the same tissue were centrifuged at the same time, interval, and speed, the tumorous cells failed to stratify, as did the normal tissue, thus indicating they possessed a higher viscosity. On the other hand, Cowdry and Paletta (1941), using the same method, but a different type of tumor cell, reported an increase in viscosity of the tumorous cell over that of the normal. From these studies, it seems probable that tumorous cells of different types, as well as from different tissues, may vary considerably in viscosity when compared to normal cells of the same organ.

Enzymes. Various enzymes have also been stratified by centrifugal force. For example, Holter and Doyle (1938) found amylase in *Amoeba proteus* to be most abundant in the layer of the mitochondria. In certain centrifuged marine eggs, Philipson (1934) found the heavier halves to contain a greater peptidase activity than did the lighter halves. However, Holter (1936) reports peptidase activity in centrifuged marine eggs to be unrelated to the granular components. In addition, eggs that are centrifuged into nucleated and non-nucleated halves demonstrate that enzymes can act independently of the nucleus.

Navez and E. B. Harvey (1935) showed that almost twice as much indophenol oxidase activity exists in the red halves of centrifuged *Arbacia* eggs as in the light halves. Shapiro (1935) reports the red halves to have a higher respiratory rate than the clear nucleated halves. On the other hand, increase of respiration due to p-phenylene diamine is more marked in the lighter than in the heavier halves (Boell, Chambers, Glancy, and Stern, 1940).

High centrifugal force produces a marked reduction in the oxygen con-

sumption of actively developing grasshopper embryos but has little effect on the oxygen consumption of diapause embryos (Bodine and Boell, 1936). Likewise, stratification of the *Ascaris* eggs produces a marked depression in oxygen consumption, even though the eggs are not killed by this procedure. Evidence is presented that respiration is depressed as a result of the disorganizing effect of ultracentrifuging (Huff and Boell, 1936).

Diminisher Substance in Ascaris. The factors responsible for chromosome diminution in *Ascaris equorum* are thought to reside in the cytoplasm (Boveri, 1910; Hogue, 1910). King and Beams (1938) reported that ultracentrifuging inhibits cytokinesis but does not stop karyokinesis or the formation of the diminisher substance. Accordingly, when the concentration of the diminisher is sufficiently great, all of the chromosomes undergo diminution. Larvae have been observed without the characteristic large germ cells.

Organizer Substance in Amphibia. It has been reported by several investigators that centrifuging the amphibian egg and early embryos in stages up to and including the gastrula gives rise to various types of abnormal development. Embryos with abnormal heads, tails, brains, eyes, *etc.*, have been produced by this method; (Hertwig, 1904; Morgan, 1906; Jenkinson, 1915; Pasquini and Reverberi, 1929; Beams, King, and Risley, 1934; Schechtman, 1937; Beams and King, 1938c; Pastells, 1940; Torrey and Breneman, 1941).

There is some evidence, especially in those embryos centrifuged in the gastrula stage, that a displacement of the organizer substance has occurred which is directly responsible for some of the abnormalities observed.

Polarity and Bilaterality. The earlier works seem to show that centrifugal force has little effect upon determining the polarity of uncleaved eggs (*cf.* Morgan, 1927, for extensive literature). Accordingly, polarity cannot be directly related to any of the protoplasmic materials that are stratifiable by the magnitude of the centrifugal force and the periods of its duration used in the earlier experiments. It must inhere, therefore, in the protoplasmic organization unaffected by relatively low centrifugal force. This would seem to be further substantiated from the fact that *Arbacia* eggs may be separated into four parts, each containing materials of different relative density, yet each quarter egg is capable of development (Harvey, 1932). Thus, Lillie (1906) concludes that "polarity is a property of the ground substance of the protoplasm." A similar conclusion was later reached by Lillie (1909a) concerning the property of bilaterality, since it, too, was unaffected in centrifuged *Chaetopterus* eggs. Conklin (1910) states that polarity and bilaterality must be associated with a "tenuous framework" which interpenetrated the entire cell and which is not disturbed by the shifting of the visible substance of the egg. He later (1917) identifies the "tenuous framework" with a "viscid spongioplasm." A fundamentally similar view has been expressed by Taylor (1931).

Beams (1936) reported that ultracentrifuging had little effect upon the polarity (formation of the rhizoid) in the eggs of the marine alga, *Fucus serratus*. The eggs were centrifuged in rather heavily populated cultures and allowed to develop in the dark. More recently, Whitaker (1937, 1940)

has studied intensively the effects of various physico-chemical factors, including ultracentrifuging, upon the polarity of a closely related species of *Fucus*, namely, *Fucus furcatus*. Whitaker found, as regards the effect of centrifuging upon the egg, that: (1) "the group effect" of crowded cultures is superimposed on the stratification effect so that, under these conditions, his findings on polarity are similar to those reported by Beams; (2) centrifuged eggs reared singly, however, or in thin cultures at a pH of 7.8 to 8.1, showed 99 to 100 per cent forming rhizoids on their centrifugal halves; (3) at pH 6.0 to 8.0, the response of the stratified eggs is intermediate, *i.e.*, rhizoids form at random; and (4) in acidified sea water (pH 6.0), the developmental response to stratification is reversed, so that now 90 per cent of the eggs form rhizoids upon their centripetal halves. Whitaker suggests that the stratification had in some way affected the pH gradient, which is the main factor directly responsible for determining the polarity of the egg.

When centrifuged at 20,000 times gravity, about 70 per cent of the pollen tubes of *Vinca rosea* appear on the centrifugal side of the cells. In those tubes which appear at other than the centrifugal pole, further growth is directed by the centrifugal force. Displacement of auxins or auxin-like substances, enzymes, or substrate or the establishment of a physiological gradient by stratification are possible explanations of these results (Beams and King, 1944). Further references concerning the effects of centrifuging upon polarity and growth in plants are given by Beams and King (1939) and by Bloch (1943).

Pease (1938, 1939) has studied bilateral determination in the eggs of *Dendraster* that were subjected to centrifugal force of the order of 45,000 times gravity. He assumes that in the normal egg there is a "ventral determinant" present as a gradient, probably in the cortical layers of the vegetative hemisphere, which determines the presumptive ventral side at the region of greatest concentration or activity. This gradient of the "determinant" is not shifted by the highest centrifugal force. Pease concludes, however, that a "substrate" is displaced to the centripetal end, at which position the ventral side of the centrifuged egg is determined. Thus, "The ventral side of the centrifuged eggs is determined by the region of the greatest interactivity of these two factors causing a partial and variable rotation of the presumptive dorsoventral axis." A review of papers by Runnstrom and by Lindahl dealing with the same subject is given by Pease.

Mitotic Figure. It has frequently been observed that centrifuged dividing eggs have their mitotic spindles displaced, often distorted, and usually oriented at right angles to the centrifugal field (Andrews, 1915; Lillie, 1909b; Morgan, 1910; Spooner, 1911; Schade, 1930; Schrader, 1934; Harvey, 1935; Luyet, 1935; Pfeiffer, 1938; and others).

The position taken up by the spindle depends upon its relative specific gravity, as compared to that of the surrounding cytoplasm and inclusions. The chromosomes are usually the heaviest component of the amphiaster and the poles of the spindle the lightest. Accordingly, the centrifuged metaphase spindle is often bent into a U shape.

Heilbrunn (1928) reports that centrifuged dividing eggs of *Arbacia* often assume irregular shapes, which he interprets as due to a pull of the firm

gelatinous but elastic asters on the membrane of the egg. He suggests that the pull of the asters on the surface may have considerable importance in the normal division in these eggs.

On the other hand, E. B. Harvey (1935) has reported that the mitotic figure of *Parechinus microtuberculatus* may be shifted by centrifuging to the centripetal pole and that the astral rays then become much elongated toward the centrifugal pole. In other batches of eggs, the reverse conditions may occur. Her observations support the view that the asters are regions of gelation. She also made the interesting observation that the first cleavage plane (primary) of centrifuged eggs is correlated with the position of the nucleus or amphiaster before centrifuging and may bear no relation to the position of the displaced spindle. In fact, the first cleavage may cut through the body of the displaced amphiaster. In addition to the primary plane of cleavage, a second plane may appear (induced plane) which is correlated with the position of the displaced spindle in the egg. Sometimes, both planes appear at the same time and either one or both may become functional.

With the exception of the paper by Schrader (1934), most of the other investigators have made no special effort to analyze the effects upon the various spindle components. By observing the spindle in centrifuged eggs of certain Heteroptera, Mollusca, and Crustacea, Schrader concludes that the fibers of the half spindle may be bent and distorted, not only as a group, but independently of each other. Accordingly, the half spindle elements are considered real structures in the living cell and not simple coagulant artifacts. Further evidence was obtained to show that the half spindles and interzonal fibers are discrete structures.

Beams and King (1936) centrifuged dividing chick embryonic cells at 150,000 times gravity and found that, in some cells, the chromosomes were completely pulled away from the spindle fibers, leaving only fragments attached to the U-shaped spindle halves. They further observed divided spindles, multipolar spindles, partially liquefied spindles and spindles with detached or broken chromosomes.

Similar observations were made on the ultracentrifuged dividing cells of the root tip of wheat (Beams and King, 1938a). They found metaphase spindles which were greatly distorted and that sometimes the chromosomes were thrown free and displaced to the centrifugal pole. Cells centrifuged while in the anaphase or telophase, before the cell plate is well developed, may have the chromosomes or the developing nuclei displaced to the centrifugal end, and the "spindle of cytokinesis," or developing phragmoplast, displaced to the centripetal end. This condition usually gives rise to a binucleate cell. Sometimes, several such nuclear mitoses may occur without division of the cytoplasm. Upon recovery, they often show multipolar spindles and heteroploidy. If the cell plate is sufficiently well formed, it is not displaced but may be ruptured by the centripetally displaced nucleus. From such studies, it is suggested that, during the early stages of development, the cell plate-forming substance may be displaced in much the same manner as is the cleavage substance in centrifuged *Ascaris* eggs, resulting in both cases in the failure of normal partition membranes to form.

Kostoff (1938) also has observed many types of abnormal spindles follow-

ing centrifuging at 10,000 times gravity. Of considerable significance are the observations of Pfeiffer (1938), who subjected the dividing eggs of *Phynchelmis* to 4,000 times gravity in a centrifuge microscope with a polarized light attachment and noted the structural changes in the spindle. The greater the spindle displaced and stretched by the centrifuging, the more pronounced was its double refraction. He suggests that the increase in double refraction may be due to the more regular orientation of the micelles by stretching.

In general, the evidence from the centrifuging on the spindle seems for the most part to support the view that it is a gelled region composed of fibers which are highly elastic.

Cleavage Inhibition. It has long been known that the cleavage of the cytoplasm can be stopped by many external agencies (cold, lipoid solvents, hypertonic and hypotonic solutions, colchicine, mechanical agitation, hydrostatic pressure, *etc.*). Furthermore, it is also true that cytoplasmic division may take place in the absence of nuclear material (E. B. Harvey, 1936). It is a rather striking fact that many cells will divide while being centrifuged (Boveri, 1910; Pfeiffer, 1938; and others).

When the fertilized eggs of *Ascaris equorum* or *Ascaris suum* are centrifuged at 150,000 times gravity, they are immediately stratified into the following layers: (1) fat at the centripetal pole, (2) clear vacuoles, (3) optically clear cytoplasm, except for the pronuclei or mitotic spindle, and (4) brown granules at the centrifugal pole. In some cases, the fat and brown granules are completely cut off from the remainder of the egg. When centrifuged with sufficient rapidity for long periods of time, mitotic divisions of the nucleus may occur without accompanying division of the cytoplasm. When removed from the centrifuge, such eggs often show on their surface pseudopodia-like processes containing clear cytoplasm. Sometimes, these are cut off from the egg and undergo active amoeboid movement in the paravitelline space. Although no measurements have been made, it would appear that, as the eggs undergo nuclear division without cleavage, an increase in the volume of the hyaline layer occurs. It might be that, under such strong centrifugal force, a "cleavage substance" becomes stratified in the clear hyaloplasmic zone and displaced from the regions where the cleavage furrows would normally form (Moore, 1938), or that there is a disruption of the sol-gel relations in the cortex, inhibiting cleavage there. Whatever the explanation may be, it seems unlikely that the failure to cleave in these cases is due to a simple packing of granules, since, in *Ascaris suum*, only the clear cytoplasmic layer cleaves at 150,000 times gravity (this force usually inhibits cleavage in *Ascaris equorum*), while at higher forces (350,000 times gravity) it too fails to cleave (Beams and King, 1937, 1940).

The changes in viscosity which are associated with the formation of the typical mitotic spindle have not been inhibited. Pease and Marsland (1939) have demonstrated that this arrest of cytokinesis is not due to hydrostatic pressure developed in the ultracentrifuge, since *Ascaris* cleavage is relatively unaffected by a hydrostatic pressure of 800 atmospheres. Moore (1938) has observed somewhat similar occurrences in the centrifuged eggs

of the sand dollar. Stratified eggs form many blastomeres at the heavy end, but the light end (containing the nucleus) may cleave into only a few large blastomeres or may not cleave at all, although the nucleus may divide. Moore has interpreted these results, too, as due to a displacement of a cleavage substance to the heavy end of the cell, where the cleavages are the most numerous.

Costello (1940) reports that fragments of unfertilized *Nereis* eggs may be obtained by elongation of the egg by high centrifugal force and subsequently cutting it into two parts. He observed that either or both of the fragments may be activated by spermatozoa or parthenogenetic agents. However, only the centripetal fragment (containing the germinal vesicle) cleaves. Costello interprets these results to mean that materials from the germinal vesicle are essential for effective fertilization and cleavage.

A complete explanation for the inhibition of cytokinesis by centrifuging while nuclear division occurs is difficult. Since the initial cleavage furrows occur at the surface, and since cleavage is known to involve a sol-gel change in the cytoplasm, undoubtedly the centrifuging has in some way affected these processes. Furthermore, the flowing of the cortical cytoplasm, which Spek (1918) has described as occurring in nematode eggs during division, has undoubtedly been affected.

Ultrastructure of Protoplasm. Since the application of polarized light, X-ray diffraction, and other ultramicroscopic methods to the study of biological materials, much interest has been centered on the possible ultramicroscopic structure of protoplasm. In fact, several structures, such as the mitotic spindle, cell membrane, *etc.*, have been found to have sufficiently ordered arrangement of their molecules to give characteristic diffraction patterns. It has been assumed that the normal spatial relationship of these molecules or micelles is in some way fundamental to protoplasm. From the important work of Svedberg and others on the molecular size and stratification of many non-living proteins in strong centrifugal fields and that of Wyckoff (1937a, 1937b), Bauer and Pickels (1937), Stanley (1939), Stern (1943), and others on various virus and agents causing tumors and other diseases, it seems not unreasonable to suppose that a similar sedimentation or stratification of the ultramicroscopic structure might take place in protoplasm.

In fact, Moore (1935) found that centrifuging *Plasmodium* for five minutes at 75,000 times gravity stopped proliferation and stratified the cytoplasm into relatively light and heavy components. He suggests that "a minimum physical essential for the life processes in *Plasmodium* is that substances of both low and high specific gravity must be in intimate association." Neither of these substances, according to Moore, can exhibit life phenomena alone.

However, *Ascaris suum* eggs were exposed to approximately 900,000 times gravity for intervals of 1 to 10 hours without killing them. Other types of cells, too, have been able to withstand high centrifugal force (400,000 times gravity) for relatively long intervals (Guyer and Claus, 1936, cancer cells; MacDougald, Beams and King, 1937, chick embryonic cells; Dornfeld,

1936, 1939, pituitary gland and adrenal gland cells). In these experiments, much greater force has been applied to the cells than was used by other investigators in the study of the molecular weights of protein, *etc.* It seems unlikely, however, that a disruption of the fundamental ultramicroscopic structure has occurred in the protoplasm, although it is conceivable that the "brush heap" arrangement of the molecules has been greatly compressed and distorted.

Recently (1944), Monné has reported that sea urchin eggs exposed to relatively low centrifugal force and subsequently examined by polarized light show a distinct birefringence within the clear layer of the ground cytoplasm. He noted that in the hyaline layer many threads or systems of concentric lamellae appear. The fibers orient at random and are always negatively birefringent in the longitudinal axis. The concentric lamellae are always positively birefringent in the radial direction. Monné observed no change in the cortex upon centrifuging, however, and concurs in the view that polarity is determined by the structure of the cortex. It would be of considerable interest to examine ultracentrifuged eggs by this method.

In this connection, too, it should be noted that Harvey and Marsland (1932) have watched, by means of the microscope centrifuge, the effect of centrifugal force upon the movement of heavy crystals in *Amoeba dubia* and have noted that "the crystals always fall in 'jerks' even when moving through a visibly clear field." May not this "jerkiness" of the crystals, observed falling through an optically structureless cytoplasm, be due to its ultramicroscopic framework?

Northen and Northen (1938) report that displacing the chloroplasts of *Spirogyra* to one end of the cell by centrifuging and subsequently reversing the cell in the centrifuge and displacing them to the opposite end results in a gradual breakdown of the "brush heap" structure of the protoplasm and a lowering of the viscosity of the cell as a whole.

Finally, it should be mentioned that the displacement of the structural elements of a living cell by forces thousands of times the force of gravity must result in considerable disturbance in its normal physiology. For, as pointed out by Mast (1926), Hofmeister long ago stated that one of the most outstanding characteristics of a living system is the fact that, within a given cell, there occur simultaneously, side by side, a multifarious aggregate of different processes, and this is possible only on the assumption that the cell is divided into numerous partially isolated subsystems containing different mixtures of chemical compounds, making it possible for a given process to occur in one compartment and a radically different process in an adjoining compartment. Yet, these systems, deranged as they undoubtedly are by high centrifugal force, proceed to function in *Ascaris* eggs sufficiently to maintain life for 10 days in a centrifugal field of 400,000 times gravity.

Bibliography

Andrews, F. M. 1915. Die Wirkung der Zentrifugalkraft auf Pflanzen. Jahrb. wiss. Bot. **56:** 221

Bauer, J. H. & E. G. Pickels. 1937. An improved air-driven type of ultracentrifuge for molecular sedimentation. J. Exp. Med. **65:** 565.

Beams, H. W. 1937. The air turbine ultracentrifuge, together with some results upon the ultracentrifuging the eggs of *Fucus serratus*. J. Mar. Biol. Assoc. United Kingdom **21:** 571.

Beams, H. W. 1947. Stratification of the erythrocytes of man by ultracentrifuging. Proc. Soc. Exp. Biol. & Med. **66:** 373.

Beams, H. W. 1948. The effects of ultracentrifuging upon the meiotic chromosomes of the male grasshopper, *Melanoplus differentialis*. J. Morph. **83:** 87.

Beams, H. W., J. B. Gatenby, & J. A. Muliyil. 1936. Ultracentrifuging the spermatocytes of *Helix aspersa*. Quart. J. Micr. Sci. **78:** 387.

Beams, H. W. & E. B. Hines. 1944. Stratification of the rat erythrocyte by ultracentrifuging. Anat. Rec. **90:** 155.

Beams, H. W. & R. L. King. 1934a. The effects of ultracentrifuging upon the Golgi apparatus in the uterine gland cells. Anat. Rec. **59:** 363.

Beams, H. W. & R. L. King. 1934b. Effect of ultracentrifuging on the mitochrondria of the hepatic cells of the rat. Anat. Rec. **59:** 395.

Beams, H. W. & R. L. King. 1935a. The effects of ultracentrifuging the spinal ganglion cells of the rat, with special reference to Nissl bodies. J. Comp. Neur. **61:** 175.

Beams, H. W. & R. L. King. 1935b. The effect of ultracentrifuging on the cells of the root tip of the bean (*Phaseolus vulgaris*). Proc. Roy. Soc. B. **118:** 264.

Beams, H. W. & R. L. King. 1936. The effect of ultracentrifuging upon chick embryonic cells, with special reference to the "resting" nucleus and the mitotic spindle. Biol. Bull. **71:** 188.

Beams, H. W. & R. L. King. 1937. The suppression of cleavage in *Ascaris* eggs by ultracentrifuging. Biol. Bull. **73:** 99.

Beams, H. W. & R. L. King. 1938a. An experimental study on mitosis in the somatic cells of wheat. Biol. Bull. **75:** 189.

Beams, H. W. & R. L. King. 1938b. A study in the cytoplasmic components and inclusions of the developing guinea pig egg. Cytologia **8:** 353.

Beams, H. W. & R. L. King. 1938c. Pigmentation changes in tadpoles of *Rana pipiens* following centrifugation during the early gastrula. J. Morph. **63:** 477.

Beams, H. W. & R. L. King. 1939. The effect of centrifugation on plant cells. Bot. Rev. **5:** 132.

Beams, H. W. & R. L. King. 1940. The air driven ultracentrifuge, its application in biology, together with some effects of ultracentrifuging the eggs of *Ascaris suum*. J. Roy. Micr. Soc. **60:** 240.

Beams, H. W. & R. L. King. 1941. Some physical properties of the protoplasm of the protozoa. Protozoa in Biological Research. Edited by G. N. Calkins and F. M. Summers.

Beams, H. W. & R. L. King. 1944. Effect of ultracentrifuging on polarity in the pollen grains of *Vinca rosea*. J. Cell & Comp. Physiol. **24:** 109.

Beams, H. W. & R. L. King. 1945. Fragmentation of amphibian erythrocytes in the ultracentrifuge. J. Morph. **77:** 63.

Beams, H. W. & H. W. Kirshenblit. 1940. Ultracentrifugation of rat spinal ganglion cells, with special reference to neurofibrillae. Anat. Rec. **76:** 95.

Beams, H. W. & J. B. Sheehan. 1941. The yolk-nucleus complex of the human ovum. Anat. Rec. **81:** 545.

Beams, J. W. 1930. An apparatus for obtaining high speeds of rotation. Rev. Sci. Instr. **1:** 667.

Beams, J. W. 1937. High rotational speeds. J. Appl. Phys., **8:** 795.

Beams, J. W. 1938. High speed centrifuging. Rev. Mod. Phys. **10:** 245.

Beams, J. W. 1947. High centrifugal fields. Wash. Acad. Sci. **37:** 221.

Beams, J. W. & A. J. Weed. 1931. A simple ultracentrifuge. Science **74:** 44.

Beams, J. W., A. J. Weed & E. G. Pickels. 1933. The ultracentrifuge. Science **78:** 338.

Bensley, R. R. 1937. On the fat distribution in mitochondria of the guinea pig liver. Anat. Rec. **69:** 341.

Bensley, R. R. 1942. Chemical structure of cytoplasm. Science **96:** 389.

Bensley, R. R. & N. L. Hoerr. 1934. Studies on cell structure by the freezing-drying method. V. The chemical basis of the organization of the cell. Anat. Rec. **60:** 251.

Bloch, R. 1943. Polarity in plants. Bot. Rev. **9:** 261.

Bodine, J. H. & E. J. Boell. 1936. The effect of ultracentrifuging on the respiratory activity of developing and blocked embryonic cells (*Orthoptera*). J. Cell. & Comp. Physiol. **7:** 455.

Boell, E. J., R. Chambers, E. A. Glancy, & K. G. Stern. 1940. Oxidase activity and respiration of cells and cell-fragments. Biol. Bull. **79:** 352.

Bourne, B. 1942. Mitochondria and Golgi apparatus. Chap. 4. Cytology and cell physiology. Edited by B. Bourne.
Boveri, TH. 1910. Uber die Teilung centrifugierter Eir von Ascaris megalocephala. Arch. Entw.-Mech. **30:** 101.
Bowen, R. H. 1928. Studies on the structure of plant protoplasm. I. The osmiophilic platelets. Zeit. f. Zellf. u. mikr. Anat. **6:** 689.
Browne, E. N. 1914. The effects of centrifuging the spermatocyte cells of *Notonecta*, with special reference to the mitochrondria. J. Exp. Zool. **17:** 337.
Claude, A. 1940. Particulate components of normal and tumor cells. Science **91:** 77.
Claude, A. 1943. Distribution of nucleic acids in the cell and the morphological constitution of cytoplasm. Biol. Symposia **10:** 111.
Clement, A. C. 1938. The structure and development of centrifuged eggs and egg fragments of *Physa heterostropha*. J. Exp. Zool. **79:** 435.
Conklin, E. G. 1910. The effects of centrifugal force on the organization and development of the eggs of fresh water pulmonates. J. Exp. Zool. **9:** 417.
Conklin, E. G. 1917. Effects of centrifugal force on the structure and development of the eggs of *Crepidula*. J. Exp. Zool. **22:** 311.
Conklin, E. G. 1931. The development of centrifuged eggs of ascidians. J. Exp. Zool. **60:** 1.
Costello, D. P. 1935. Fertilization membranes of centrifuged *Asterias* eggs. I. The effects of centrifuging before fertilization. Physiol. Zool. **8:** 65.
Costello, D. P. 1939. The volumes occupied by the formed cytoplasmic components in marine eggs. Physiol. Zool. **12:** 13.
Costello, D. P. 1940. The fertilizability of nucleated and non-nucleated fragments of centrifuged *Nereis* eggs. J. Morph. **66:** 99.
Cowdry, E. V. & F. X. Paletta. 1941. Alterations in nuclear viscosity during experimental carcinogenesis determined by ultracentrifugation. Amer. J. Path. **17:** 335.
Daniels, M. I. 1938. A cytological study of the Gregarine parasites of *Tenebrio molitor*, using the ultracentrifuge. Quart. J. Micro. Sci. **80:** 293.
Dornfeld, E. J. 1936. Nuclear and cytoplasmic phenomena in the centrifuged adrenal gland of the albino rat. Anat. Rec. **65:** 403.
Dornfeld, E. J. 1939. The ultracentrifuge in cellular biology. Marquette Med. Rev. **3:** 51.
Gatenby, J. B. & T. A. A. Moussa. 1949. The cytoplasmic inclusions of the spermatocyte of *Limnaea stagnalis*. La Cellule **52** (3): 297.
Gray, J. 1931. A Textbook of Experimental Cytology.
Green, D. E. 1949. Enzymes in teams. Scientific American **181:** 48.
Guyer, M. F. & P. E. Claus. 1936a. Growth of cancerous and of embryonic tissues stratified in the ultracentrifuge. Proc. Soc. Exp. Biol. & Med. **35:** 468.
Guyer, M. F. & P. E. Claus. 1936b. Recovery changes in transplanted anterior pituitary cells stratified in the ultracentrifuge. Biol. Bull. **71:** 462.
Guyer, M. F. & P. E. Claus. 1939. Relative viscosities of tumor cells as determined by the ultracentrifuge. Ant. Rec. **73:** 17.
Guyer, M. F. & P. E. Claus. 1942. Increased viscosity of cells of induced tumors. Cancer Research **2:** 16.
Harvey, E. B. 1932. The development of half and quarter eggs of *Arbacia punctulata* and of strongly centrifuged whole eggs. Biol. Bull. **62:** 155.
Harvey, E. B. 1933. Development of the parts of sea urchin eggs separated by centrifugal force. Biol. Bull. **64:** 125.
Harvey, E. B. 1934. Effects of centrifugal force on the ectoplasmic layer and nuclei of fertilized sea urchin eggs. Biol. Bull. **66:** 228.
Harvey, E. B. 1935. The mitotic figure and cleavage plane in the egg of *Parechinus microtuberculatus*, as influenced by centrifugal force. Biol. Bull. **69:** 287.
Harvey, E. B. 1936. Parthenogenetic merogony or cleavage without nuclei in *Arbacia punctulata*. Biol. Bull. **71:** 101.
Harvey, E. N. 1931. The tension at the surface of marine eggs, especially those of the sea urchin *Arbacia*. Biol. Bull. **61:** 273.
Harvey, E. N. 1932. The microscope-centrifuge and some of its applications. Franklin Inst. **214:** 1.
Harvey, E. N. 1934. The air turbine for high speed centrifuging of biological material, together with some observations on centrifuged eggs. Biol. Bull. **66:** 48.
Harvey, E. N. 1938. Some physical properties of protoplasm. J. Appl. Phys. **9:** 67.
Harvey, E. N. 1946. Can the sex of mammalian offspring be controlled? J. Hered. **37:** 71.

Harvey, E. N. & A. L. Loomis. 1930. A microscope-centrifuge. Science **72:** 42.
Harvey, E. N. & D. A. Marsland. 1932. The tension at the surface of *Amoeba dubia* with direct observations on the movement of cytoplasmic particles at high centrifugal speeds. J. Cell. & Comp. Physiol. **2:** 75.
Heilbrunn, L. V. 1921. Protoplasmic viscosity changes during mitosis. J. Exp. Zool. **34:** 417.
Heilbrunn, L. V. 1928. The colloid chemistry of protoplasm. Protoplasma-Monographier I.
Henriot, E. & E. Huguenard. 1925. Sur la realisation de trés grandes vitesses de rotation. Compt. rend. **180:** 1389.
Henriot, E. & E. Huguenard. 1927. Les grandes vitesses angulaires obtenues par les rotors sans axe solide. J. Phys. Radium. **8:** 433.
Hertwig, O. 1904. Weitere Versuche uber den Einfluss der Centrifugalkraft auf die Entwicklung tierischer Eier. Arch. f. mikr. Anat. **63:** 643.
Hoerr, N. L. 1943. Methods of isolation of morphological constituents of the liver cell. Biol. Symp. **10:** 185.
Hogeboom, G., W. C. Schneider & G. E. Palade. 1948. Cytochemical studies on mammalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopical particulate material. J. Biol. Chem. **172:** 619.
Hogue, M. J. 1910. Uber die Wirkung der Centrifugalkraft auf die Eier von *Ascaris megalocephala*. Arch. f. Ent.-Mech. **29:** 109.
Holter, H. 1936. Studies of enzymatic histochemistry. J. Cell. & Comp. Physiol. **8:** 179.
Holter, H. & W. L. Doyle. 1938. Über die Lokalization der Amylase in Amöben. Compt. rend. Lab. Carlsberg. Ser. Chim., **22:** 219.
Huff, G. C. & E. J. Boell. 1936. Effect of ultracentrifuging on oxygen consumption of the eggs of *Ascaris suum*, Goeze. Proc. Soc. Biol. & Med. **34:** 626.
Jenkenson, J. W. 1914. On the relation between the structure and the development of the centrifuged egg of the frog. Quart. J. Micr. Sci. **60:** 61.
King, R. L. & H. W. Beams. 1938. An experimental study of chromatin diminution in *Ascaris*. J. Exp. Zool. **77:** 425.
King, R. L. & H. W. Beams. 1942. Ultracentrifugation and cytology of *Spirillum volutans*. J. Bact. **44:** 597.
Knight, T. A. 1806. On the direction of the radicle and germen during the vegetation of seeds. Phil. Trans. Roy. Soc. London, Part I **96:** 99.
Kostoff, D. 1937. Chromosome alterations by centrifuging. Science **86:** 101.
Kostoff, D. 1938. The effect of centrifuging upon the germinated seeds. Cytologia **8:** 420.
Lazarow, A. 1943. The chemical structure of cytoplasm as investigated in Professor Bensley's laboratory during the past ten years. Biol. Symp. **10:** 9.
Lillie, F. R. 1906. Observations and experiments concerning the elementary phenomena of embryonic development in *Chaetopterus*. J. Exp. Zool. **3:** 153.
Lillie, F. R. 1909a. Polarity and bilaterality of the annelid egg. Experiments with centrifugal force. Biol. Bull. **16:** 54.
Lillie, F. R. 1909b. Karyokinetic figures of centrifuged eggs: an experimental test of the center of force hypothesis. Biol. Bull. **17:** 101.
Lucas, A. M. & W. W. Herrmann. 1935. Effect of centrifugation on herpetic intranuclear inclusions with a note on cytoplasmic inclusions of unknown origin in the rabbit cornea. Am. J. Path. **11:** 969.
Luyet, B. J. 1935. Behavior of the spindle fibers in centrifuged cells. Proc. Soc. Exper. Biol. & Med. **33:** 163.
MacDougald, T. J., H. W. Beams, & R. L. King. 1937. Growth of ultracentrifuged cells in tissue culture. Proc. Soc. Exper. Biol. & Med. **37:** 234.
Mast, S. O. 1926. The structure of protoplasm in *Amoeba*. Amer. Natur. **60:** 133.
Monné, L. 1944. Cytoplasmic structure and cleavage pattern of the sea urchin egg. Arkiv. f. Zoologi. **35:** 1.
Moore, A. R. 1935. On the significance of cytoplasmic structure in *Plasmodium*. J. Cell. & Comp. Physiol. **7:** 113.
Moore, A. R. 1938. Segregation of "cleavage-substance" in the unfertilized egg of *Dendraster excentricus*. Proc. Soc. Exp. Biol. & Med. **38:** 162.
Morgan, T. H. 1906. The influence of a strong centrifugal force on the frog's egg. Arch. Ent.-Mech. **22:** 553.
Morgan, T. H. 1910. Cytological studies of centrifuged eggs. J. Exp. Zool. **9:** 593
Morgan, T. H. 1927. Experimental Embryology. New York.

Navez, A. E. & E. B. Harvey. 1935. Indophenol oxidase activity in intact and fragmented *Arbacia* eggs. Biol. Bull. **69:** 342.
Northen, H. T. & R. T. Northen. 1938. Studies of protoplasmic structure in *Spirogyra*. II. Alterations of protoplasmic elasticity. Protoplasma **31:** 9.
Palade, G. E. & A. Claude. 1949. The nature of the Golgi apparatus. I. Parallelism between Golgi apparatus and intracellular myelin figures. J. Morph. **85:** 35.
Palade, G. E. & A. Claude. 1949. The nature of the Golgi apparatus. II. Identification of the Golgi apparatus with a complex of myelin figures. J. Morph. **85:** 71.
Pasquini, P. & G. Reverberi. 1928. Studi sulla determinozione nello sviluppo degh Anfibi. Boll. Inst. Zool. (Roma) **7:** 11.
Pasteels, J. 1939. (Cited from Needham, J. 1942. Biochemistry and Morphogenesis. Cambridge).
Patten, R. & H. W. Beams. 1936. Observations on the effect of the ultracentrifuge on some free-living flagellates. Quart. J. Micr. Sci. **78:** 615.
Pease, D. C. 1938. The influence of centrifugal force on the bilateral determination of the spirally-cleaving egg of *Urechis*. Biol. Bull. **75:** 409.
Pease, D. C. 1939. An analysis of the factors of bilateral determination in centrifuged Echinoderm embryos. J. Exp. Zool. **80:** 225.
Pease, D. C. & D. A. Marsland. 1939. The cleavage of *Ascaris* eggs under exceptionally high pressure. J. Cell. & Comp. Physiol. **14:** 407.
Pfeiffer, H. H. 1938. Double refraction measurements and structural changes in mitotic spindles disturbed by centrifugal force. Biodyn. **2:** 1.
Philipson, T. 1934. The peptidase in the eggs of *Psammechinus miliaris*. Comp. rend. Lab. Carlsberg. **20:** 4.
Pickels, E. G. 1936. Optical designs for observing objects in centrifugal fields of force. Science **83:** 471.
Rosenbush, C. T. & A. M. Lucas. 1939. Studies on the pathogenicity and cytological reactions of the submaxillary gland virus of the guinea pig. Am. J. Path. **15:** 303.
Schaede, R. 1930. Zentrifugalversuche mit Kernteilungen. Planta **11:** 243.
Schechtman, A. M. 1937. Mechanism of anomaly induction in frog eggs by means of centrifuge. Proc. Soc. Exp. Biol. & Med. **37:** 153.
Schrader, F. 1934. On the reality of spindle fibers. Biol. Bull. **67:** 519.
Schultze, O. 1894. Die künstliche Drzeugung von Doppelbildungen bei Froschlarven mit Hilfe abnormer Gravitationswirkung. Arch. Entw.-Mech. **1.**
Shapiro, H. 1935. The respiration of fragments obtained by centrifuging the egg of the sea urchin, *Arbacia punctulata*. J. Cell. & Comp. Physiol. **6:** 101.
Singh, B. N. 1938. The cytoplasmic bodies in the oogenesis of the vulture (*Neophron percnopterus ginginianus*) and the effect of ultracentrifuging on the oocytes of the pigeon. Proc. Roy. Irish Acad. **45:** 33.
Spek, J. 1918. Oberflächenspannungsdifferenzen als eine Ursache der Zellteilung. Arch. Entw.-Mech. **44:** 1.
Spooner, G. B. 1911. Embryological studies with the centrifuge. J. Exp. Zool. **10:** 23.
Stanley, W. M. 1939. Recent advances in the study of viruses. Science in Progress. Yale University Press. New Haven.
Stern, K. G. 1943. Studies on macromolecular particles endowed with specific biological activity. Biol. Symposia **10:** 227.
Svedberg, T. & K. C. Pedersen. 1940. The Ultracentrifuge. Oxford University Press.
Taylor, C. V. 1931. Polarity in normal and centrifuged eggs of *Urechis caupo fisher* and *macginitie*. Physiol. Zool. **4:** 423.
Torrey, T. W. & W. R. Breneman. 1941. Abnormalities in frog embryos induced by centrifugation. Proc. Ind. Acad. Sci. **50:** 213.

THE ACTION OF COLCHICINE ON CELL DIVISION IN HUMAN CANCER, ANIMAL, AND PLANT TISSUES*

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I. Historical Development

The meadow saffron, *Colchicum autumnale*, has been known botanically and pharmacologically since early times. The plant resembles closely the spring crocus, but, unlike this plant, juices extracted from its bulbous stem (corm) and from its seeds yield a toxic drug, colchicine. Chemically, colchicine has a phenanthrene nucleus and has been referred to as a phenanthrene alkaloid. The early history of the drug, its therapeutic usages, its chemical formula, and its isolation and crystallization have been reviewed by Lits, Kirschbaum and Strong (1938), Levine (1945), and others. A list of the publications dealing with the various phases of the colchicine problem has recently been compiled by Eigsti (1947), to which Pierre Dustin has added many titles. A considerable number of these papers dealing specifically with the effects of this drug on cancer tissue and the somatic and reproductive cells of animals and plants, has been reviewed by Dermen (1940), Krythe and Wellensiek (1942), Ludford (1945), and Levine (1945).

Here, the purpose will be to reemphasize some of the studies on the action of this drug on the mechanism of cell division and to correlate and to integrate these findings with the work done in this laboratory. The action of colchicine on the mechanism of cell division and its influence on growth has been under investigation here since 1940. This report will deal with the following: (1) the effect of a given dose of colchicine on root-tips of *Allium cepa;* (2) the action of colchicine on advanced neoplasm in man; and (3) colchicinized plant tissues as influenced by X rays. Colchicine, combined with X rays, has been initiated in the treatment of human cancer.

The Colchicine Effect

Dustin and his students first began a study of chemical agents that affect mitoses in tissues of animals. In collaboration with Grégoire (1933), the effect of sodium cacodylate, trypoflavin, and a number of unrelated organic and inorganic compounds were studied on the Crocker mouse sarcoma. Mitotic counts were made at short intervals for the first 24 hours and at two-day intervals, after the 48th hour, up to 8 days. At 24 hours, the maximum number of mitoses is reached. These studies seemed to set the method and procedures for testing the effects of various chemicals on animal cells. Lits (1934) tested the effect of colchicine on mitoses in animal cells. The effects of colchicine on the smooth muscles and peripheral and central nervous systems of man and other animals were known. Its action has been compared with muscarine, pilocarpine, and physostigmine and

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found to be alike. Dixon and Malden (1908) showed that colchicine affects the bone marrow and leucocytosis occurs in man, rat, and dog. Bone marrow cells, the authors pointed out, showed abundance of mitotic figures.

Colchicine Effect on Cell Division in Animals. Lits's studies on the white rat after subcutaneous injections of 0.5 to 0.005 mg. per 20 gm. of body weight, confirmed Dixon and Malden's blood studies. Small doses of the drug, Lits found, increased the leukocyte count fourfold, and a return to the normal number occurred in seven hours. The higher concentration induced artificial division and nuclear destruction. In Dustin's (1934) studies of mitotic poisons, colchicine became the principal agent, and the subject, the graft sarcomas on the rat. Small doses of colchicine 1/40 mg. (0.000025 mg. or 2.5 mg. per cent) were injected subcutaneously into rats with tumors measuring 1 cm. in diameter. Four hours after the injection, the number of mitoses increased from 2.1 mitoses per microscopic field to 12.05. At 9.5 hours, there were 33.4 division figures, at 25 hours, the number of mitoses declined to 16.7 per field, and, at 48 hours after the injection, the mitotic count declined to 4.75.

During the process of colchicinization, from the tenth to the twenty-sixth hour, numerous pycnotic nuclei and complete nuclear disintegration were observed. The nuclear membrane disappears, the cytoplasm becomes turgid, the chromosomes agglutinate, and mitosis is arrested in metaphase. A day after the injection, the chromosomes in metaphase are distributed irregularly throughout the cytoplasm, for spindle figures are completely lacking. Chromosomes become distinct, and achromatic formations are seen. Normal nuclear division reappears, though some mitotic figures are abnormally large. The tumor regresses and many necrotic zones are now apparent in it.

Similar nuclear reactions to colchicine were reported by Dustin in normal tissues such as the Kupfer liver cells, reticulo-endothelial cells, megocytes, and testicular tissue. In subsequent studies, Dustin (1937 and 1938) made comparative studies of the effects induced by colchicine, trypaflavine, and arsenical compounds like sodium cacodylate. Cellular reactions to colchicine, he believed, approached that of the arsenicals. These consisted of rapid excitation to mitoses in which the chromosomes were condensed. Many of these chromosome masses disintegrated. Of those that survived, an achromatic figure was developed, the chromosomes became distinct, and mitosis was achieved. However, the striking difference between sodium cacodylate and colchicine lay in the extreme differences in the concentration of the effective dose. Nuclear effects induced by sodium cacodylate are produced by less than 1:1,000,000 of colchicine. All cells in a generative zone, or cells stimulated to divide through trauma, implantation, carcinogenic agents, or hormones, are influenced by colchicine.

Ludford (1936) studied a number of substances, such as auramine, urethane, sodium cacodylate, and colchicine, through their effect on mouse tumors. He observed that the action of colchicine on cell division was essentially the same as that of sodium cacodylate, except that it was active in much greater dilution and over a much wider range of concentration.

Tissue cultures of mouse tumor 63 responded to concentration of 1:100,000 to 1:100 million. Concentrations which inhibited mitosis acted on resting, normal, and malignant cells. The cells became round, and mitochondria appeared shorter, indicating, Ludford believed, an unfavorable condition. The chromosome once formed, split, but owing to the absence of the spindle mechanism separation remained incomplete.

Ludford showed that the action of colchicine is cumulative. Tumors from animals which receive separate injections display large numbers of mitotic figures. In the final analysis, Ludford holds that colchicine exerts its influence on the cytoplasm. Ludford (1936) threw new light on the significance of the larger number of metaphases after colchicine treatment. While workers in the Dustin laboratory believed that the effect of the drug was to stimulate cell division, Ludford pointed out that the increase in number of mitoses after colchicine was due to an accumulation of arrested mitoses. Nebel and Ruttle (1938) soon came to the same conclusion.

Brues and Cohen (1936) pointed out that, in the regeneration of the rat liver, following small doses of colchicine (0.02 to 0.05 mg. per 100 gm. of body weight), the chromosomes spread apart and are readily counted in arrested metaphase stages. An appropriate dose of sodium cacodylate induced the same changes. Colchicine derivatives tested by these workers produced mitotic effects similar to those induced by colchicine, but the dosages required were considerably higher. Brues and Cohen studied the effects of colchicine in oil and in aqueous solution, and reported that colchicine in oil required a higher concentration of the drug to bring about minimal effective doses. Brues and Jackson (1937) tested the effect of colchicine on the periphery and central portions of the tumor. Metaphases were many times more numerous in the peripheral cells than in the central portions of the tumor. The latter had about the same number as the control in colchicinized tumor tissue. Surviving cells were atypical following colchicine treatment.

In tissue cultures of fibroblasts, Bucher (1939) found that colchicine exerted rhythmic changes in nuclear phenomena. At first, there is a wave of nuclear divisions, followed later by a wave of activity in which pycnosis is the predominating character. This in turn is followed by a wave of division stages. Colchicine retards the division rhythm with initial injury in two to three hours.

Paletta and Cowdry (1942) made single injections of 0.25 cc. of 0.01 per cent colchicine per 20 gm. of body weight of mice, including late embryos to adult stages, treated previously from 22 to 161 days with methylcholanthrene. Under centrifugation, the chromosome clumps and stainable cytoplasm were less uniform in carcinomata than in embryonic or hyperplastic hair follicles. No consistent difference was noted between the uncolchicinized and colchicinized tissue. That a change in viscosity had taken place was not excluded.

Gavrilov and Von Bistram's (1939) tissue cultures of chick embryo heart and iris pointed out not only that colchicine influenced the nuclear contents of the cell, but that the cytoplasm revealed vacuolization and granulation.

The intensely clumped globular mass of chromosome material was recognized as a new type of cell derived from macrophages and fibroblasts.

For the lower vertebrates, Delcourt (1939[1,2]) found that amphibia and other poikilothermal animals' reactions to given doses of colchicine were slow. Yet the nuclear changes were similar to those in the mouse. The spindles were absent and the chromosomes were condensed. In the corium of *Triturus*, Peters (1946) found, after colchicine treatment, disoriented and star-like patterns of chromosomal arrangements, which were interpreted as spindle formation recovery. Both normal and abnormal diploid mitotic figures were abundant during recovery. Hall (1946) and Samartino and Rugh (1946) studied colchicine effects on sperm and ovulation and early development of the frog. Eggs fertilized by colchicinized sperm developed abnormally. Colchicine prevented ovulation when injected into sexually mature frogs. The larvae were retarded and abnormalities occurred. Cytological observations revealed pycnotic nuclei, multinucleate cells, and that few metaphase figures were present. Hall believed that colchicine effects are due to some changes induced in the enzyme system.

Tennant and Liebow (1940) attacked the problem through the tissue culture method, using tissue of mouse breast cancer and heart tissue from newborn mice. Colchicine in concentration of 1:64 million reduced the expansion rate of colonies of the breast cancer. The rate of mitoses was found to be reduced, but the mitoses accumulated, although profound nuclear changes in the cancer cells took place, they remained viable and transplanted to animals. Ethylcarbylamine induces similar changes in the tissues, but the concentrations required were of a higher level and acted within a much narrower range. These workers found changes in the cancer cells at one hour after treatment. The chromosomes became a thick spherical mass near the center of the cell. The prophase stages at two hours were abundant. Micronuclei derived from swelling of the clumped masses of chromosomes were absent at nine hours. Many of the cells necrotized with extrusion of pycnotic and nuclear debris. Concentrations as low as 1:128 million produced similar changes after 16 hours.

Beams and Evans's (1940) interpretation of the effects of colchicine led them to expound the view that colchicine influenced the viscosity of the cytoplasm. They found that sea urchin eggs, placed in appropriate dilutions of colchicine in sea water after fertilization, stratified more easily than eggs that had developed in sea water. This, they held, indicated a lower viscosity induced by the alkaloid. Wilbur (1940) investigated the same species of sea urchin and found that certain concentrations of colchicine prevented fertilized eggs from progressing in division but that they began the metaphase stages after their return to sea water. Wilbur holds that colchicine in sea water does not appreciably alter the viscosity of unfertilized sea urchin eggs. The increase in viscosity, however, which normally follows fertilization, is prevented by colchicine.

In neuroblasts of the grasshopper, observed *in vitro* under the influence of various concentrations of colchicine by Gaulden and Carlson (1947), it was reported that spindle formation is completely prevented and the

chromosomes are scattered throughout the cell. In *Drosophila melanogaster*, colchicine exerts its effects on the larvae; the salivary gland cells seem little affected by the drug. Braungart and Ott (1942) further report that the absence of the spindle was observed and that cells with tetraploid chromosome number were common. Hadorn and Niggli (1946) exposed gonads of *Drosophila* to phenol and to colchicine. They believe that colchicine cannot be used as an efficient agent in producing gene mutations. Phenol gave mutation rates much higher than in any experiment with other chemicals. The agents were applied to extirpated mature testes of larvae and implanted after treatment.

Colchicine Effects on Cell Division in Plants. The reports of Lits and Dustin on the effects of colchicine on mitoses in animal cells led Havas (1937) to study the influence of this alkaloid on the wheat seedling. While the first effect, Havas reported, was one of stimulation of growth, the succeeding five to six days were characterized by a depressive effect, with bulbous hypertrophy of the root tip and other parts of the plant. Havas recognized cell wall thickenings and liquefication of the parenchymatous elements. Extracts of the European mistletoe (*Viscum album*), the author reported, produced similar effects, though more slowly. In a subsequent study dealing with the significance of colchicine on the growth of plants, Havas (1938) tested a number of tissues of different plants and concluded that colchicine influenced the mobilization of hormones already in the plants, specifically reduced vitamin C, and thus interfered with growth. Blakeslee (1937) showed that colchicine induced polyploidy in plants and, in an analysis of the service chemicals may render plants (1939), discussed the production of new species through the application of a chemical like colchicine. He induced chromosome doubling in 65 different kinds of plants, including varieties and species: 41 different species, 24 genera, and 14 families of flowering plants.

Gavaudan, P., *et al.* (1937) used the roots of *Allium cepa* as their test material and subjected them to an aqueous solution of colchicine 0.2–0.1 gm. per liter of water. They too observed the hypertrophy of the root tip behind the meristem after colchicine treatment, and, in sections of the tissue 40 hours after an exposure of 0.001 per cent of colchicine, noted many abnormal mitotic figures. The chromosomes in metaphase were scattered through the cells, thus revealing the similarity of effect that colchicine exercised on animal tissue. Pseudoanaphases occurred in which the chromosomes were arranged in quadrilateral or pseudotelophases, with the chromosomal groups united by bridges. Gavaudan grew *Vicia faba* in dilute solution 1:40,000 and found those exposed 226 hours produced giant cells with vacuolate cytoplasm in which some starch grains and mitochondria could be identified. Chromosome doubling was observed. Where the $2n = 12$, 120 and 160 were counted. Gavaudan believed the chromosome number is limited in these colchicinized tissues by difficulties in nutrition which may be surmounted by aseptically grown plantlets *in vitro*. Plantlets of *Vicia faba* subjected to colchicine, Gavaudan (1938) found, developed into diverse monstrosities.

Kostoff (1938) studied the effects of colchicine on germination of grains of various cereals, and seeds of a number of economic plants. His examination of these tissues revealed abnormal nuclei, as in plant galls and animal cancer, multinucleate cells, and abnormal mitoses. Similar phenomena were observed when the seeds were germinated and grown under the influence of aqueous solutions of acenaphthene.

Eigsti (1938) investigated the cytological changes induced by colchicine in *Allium cepa*, corn, and the radish. Changes induced in these tissues were found by Eigsti to depend on three factors: concentration, length of exposure to the drug, and the physiological activity of embryonic cells at the time of treatment. Eigsti's (1939) studies on the pollen tube nuclei of *Tradescantia occidentalis* and *Polygonatum commutatum* indicated chromosome breakage. In the division of the generative cells in the pollen grains of these species and in *Lilium*, the daughter chromosomes move into the same nucleus and the diploid number is retained.

Levan (1936) made careful studies of the roots grown from bulbs of the onion, *Allium cepa* and *A. fistulosum*, exposed to varying concentrations of colchicine. He examined these roots at short intervals and described the behavior of mitoses under the influence of colchicine as "C-mitosis." The effects of various concentrations were studied and Levan showed that 0.0055 per cent solution of this alkaloid caused spindle disturbances. Levan described the swellings at the treated root tips as tumors and claimed that these were caused by an increase in volume of the meristematic cells. Gavaudan held that the tissue behind the tip forms these swellings. This zone is not well defined, for it includes parts of the meristem and cells about to differentiate to form the various tissues of the root. Levan stated that the formation of new cells was suppressed. Doubling of chromosomes was studied with relation to the length of time the roots were immersed in colchicine and its concentrations. At four hours and exposures with concentration of 0.01, 0.05, and 0.1 per cent, the cells showed C-mitosis. Forty-eight hours after exposure and return to water, onion root tip reconstruction of nuclei began. In the first meiotic division of various species of *Allium*, under the influence of colchicine, the bivalent chromosomes separate immediately, Levan (1939) pointed out, and produce a tetraploid chromosome number. In the root-tip cells, the chromatids fail to separate at the centromere at anaphase. Levan (1938) says that the chromatids assume "x"-shaped form due to failure of division at the spindle attachments. Nebel (1930) studied polyploidization under the influence of colchicine. Inhibition of anaphase stages resulted in polyploid nuclei which originate through the restitution of an interphase nucleus from metaphase chromosomes.

Shimamura (1939) followed the cytological changes induced by colchicine in *Allium cepa* and two species of *Lycopersicum*. The growing points of young plants of the tomato were treated with a colchicine-lanoline paste. Cytologically, the observation revealed confirmation of Levan's studies. Berger and Witkus (1943) studied root tips of *Spinacia* and *Allium* seedlings and, as pointed out by Shimamura, unorganized spindle substance is produced, which takes the form of an achromatic sphere about the 'diplochromosomes' during metaphase. In division, Shimamura described the

substance as forming dumbbell-shaped structures and so brought about nuclear division. In spinach, the achromatic granular substance is lacking and the chromosome forms into a dense clump. Wada (1940), on the basis of *in vivo* observations, reported the effect of the drug on the stamen cells of *Tradescantia*. The colloid changes of the spindle were studied, and the author assumes that colchicine acts as a surface action substance in the dividing cell and lowers the surface tension of the attractoplasm. The breakdown of this substance begins simultaneously with the reduction of the chromosome. This substance finally develops into a liquid mass of plasm full of granules and flows together with the cytoplasm of the mother cell.

The chromosomal behavior in onion root-tip cells under the influence of colchicine was investigated by D'Amato (1948). His figures of partial colchicine mitoses show evidence of chromosomal failure to separate, due to "stickiness" of chromosomes and spindle disturbances. Östergren (1943) explained the separation of the chromatids on the basis of a mutual repulsion localized at the centromere.

In the lower plants, colchicine action is similar to that in higher plants. In the hepatic, *Pallavicinia lyselii*, Wolcott exposed the gametophyte to colchicine for 5 to 48 hours. Inhibited spindle formations in the spore mother cell undergoing reduction divisions and nuclei with double chromosome numbers resulted. The chromosomes were clumped and elators showed suppression of the spiral thickenings. Failure of cleavage or furrowing in the spore mother cell is described as an effect of colchicine on the cytoplasm and independent of its action on the spindle mechanism. The action of this alkaloid on the spores, young gametophytes, and sporophylls of ferns, Rosendahl (1941) reported, induced polyploidy, increased the size of cells, and reduced the frequency of cell division. Haploid prothallia grew more vigorously than polyploid ones.

From an extensive review of the available literature, far greater than is indicated above, where the emphasis was directed to the mechanics of cell division as influenced by colchicine, the evidence is clear that this plant extract exerts an influence on the actively growing tissue of a large number of animals and plants. The influence is predominantly on the achromatic substance of the cell; *i.e.*, the spindle fibers apparently necessary to normal chromosome division fail to develop. Yet the granular cytoplasmic substance from which the spindle arises may or may not be present. This phenomenon was never carefully observed in meristematic tissue of the plant. The chromosome, while showing changes such as shortening, clumping, and "sticking," frequently remains intact after applications of low concentrations of the drug for short duration. Yet the chromosomes scattered throughout the cytoplasm of the cell show separation of the chromatids, although adhering tenaciously at the centromere. At this point, too, the chromatids finally separate and their cells possess a double number of chromosomes. The nucleus is reconstituted as a giant, and frequently the chromatin material is divided to reconstitute two or more nuclei in a giant cell. These nuclei may frequently be of different sizes.

Under the influence of larger doses of colchicine, or with longer exposures

to the drug, the dividing nuclei not only fail to produce the spindle, but the chromatic material becomes pycnotic and the degeneration of the cell proceeds. The arrest of nuclear division of animal and plant cells in the metaphase stage made this drug a useful tool for the study of genetics and endocrinology. The arrested mitoses associated with leukocytosis and hemorrhage, and attested by the interruption of the growth processes of tumors, led a number of investigators to explore the value of colchicine as a therapeutic agent in the treatment of cancer.

Colchicine in Cancer and Crown Gall. Amoroso (1935) noted the beneficial effects colchicine had on the malignant growths of patients whom he treated primarily to relieve acute attacks of gout. To establish the validity of these observations, Amoroso set up a series of experiments in which he studied the effects of this drug on cancer in mice. Grafts of mouse carcinoma 63 and one case of epithelioma of the mouth of a dog regressed after small subcutaneous injections of this alkaloid. Similar studies were made on a representative number of animal tumors with indifferent results. Regression was generally followed by resumption of tumor growth.

Dustin (1934) studied the action of colchicine on sarcoma grafts and presented observations indicating the possibility of the use of the drug in the treatment of cancer.

Lits, Kirschbaum, and Strong (1938[1, 2]) studied colchicine effects on malignant lymphoid neoplasms in mice of two different strains. Regression of tumors occurred, with the number of survival days almost doubled. Tumor growth was not permanently impaired.

Peyron *et al.* (1936 to 1937) tested the effects of repeated injections of colchicine over a period of three to four weeks, on Shope papilloma, and found the tumors regressed completely. In another series of tests, rabbits with papilloma on both flanks of the experimental animals were used. The growths on one side were treated with an aqueous solution and an ointment containing colchicine. The other flank served as control. Regression was rapid on the treated side. The control flanks showed some regression and remained stationary, or some grew and became malignant.

Brown (1939) and Derman (1940) influenced growth of crown gall disease by application of colchicine. These experiments were carried out in two phases: one in which the treatment was applied to fully developed galls, and the other in which prevention of development of the tumor by the use of the alkaloid was studied. Havas (1937), who earlier studied the influence of colchicine on crown gall, did not treat the developing tumor locally, but tried to influence its growth through the sap movement in the stem. Both experimental methods showed inhibition of growth of the tumor and destruction of fully grown galls through the action of colchicine.

Solacolú, Constantinesco, and Constantinesco (1939) covered crown galls on the geranium and hypocotyls of the castor bean with a lanolin paste which incorporated 0.5 to 0.75 per cent of colchicine, three or four times at fifteen-day intervals. These overgrowths, though small, were completely arrested, and microscopic examination of the tissue revealed the presence of a new suberized tissue which completely enucleated the growth from the normal tissue and interfered with its nutrition.

The therapeutic value of colchicine as a growth arresting agent in malignancy or normal tissue is now generally denied. While growth of animal tissue may be retarded or delayed, the effects of the drug are ultimately dissipated and growth is resumed. The continued use of the drug to prolong the period of arrest causes lethal poisoning.

Colchicine and X Rays

The arrest of nuclear divisions in the metaphase stage of normal and malignant tissues, through the action of colchicine, made possible another therapeutic measure to control growth. Roentgenologists have contended that the dividing cell is more vulnerable to the ionizing effects of radium emanation and X rays than the resting cell. The simultaneous exposition of the chromosomes in an increasing number of cells has made the use of colchicine in combination with X rays a subject for further investigation.

The Mitotic Index. Studies on the effects of colchicine in combination with X rays are still limited in number, although some intensive work has been done with these agents on tumors in rats and in a small number of isolated cases of human cancer. The mechanism of cell division in the metaphase stage, interrupted by colchicine treatment, has made this drug, to the extent of our present knowledge, the most favorable agent for use in combination with X rays.

The application of X rays to colchicinized tissue required, however, a more accurate knowledge of the cytological status of the chemically treated tissue. Exposing the cells to colchicine and counting the number of nuclei at various intervals after the administration of this drug offered the only means of ascertaining the facts. Metaphases after colchicine have been counted by Dustin and others previously with the purpose of demonstrating the supposed stimulatory action of the drug on cell division. Ludford (1936) and Tennant and Liebow (1940) showed there was no stimulation, but that the nuclear divisions were arrested in metaphase and began to accumulate. The data collected showed, however, that the number of metaphases increased for a time after the injection of the chemical.

Oughterson, Tennant, and Hirshfeld (1937) reported such studies on human cancer cases. Of 21 patients who received the drug (1 to 4 mg.) subcutaneously or intra-muscularly, 15 were biopsied before and after the injection. In eleven cases, arrested mitoses in metaphase were observed. In one case, a biopsy was made before the colchicine injection and again at 5, 9½, and 12 hours afterward. At the 12th hour, the entire tumor was removed. The mitotic count after colchicine showed an increase of 700 per cent. These authors concluded that the colchicine technique made it possible to obtain a more accurate index of the rate of growth of a tumor than can be obtained by ordinary methods. Du Bilier and Warren studied the mitotic activity of the Brown-Pearce rabbit epithelioma to determine the sequence of events in this tumor following single and multiple doses of colchicine. Small biopsies were made before and 5 to 7 hours after colchicine injection, and the longest period before removing a tumor was 31 hours. Three thousand cells were counted per biopsy.

The number of mitotic figures after 5 to 7 hours showed a larger count for

the lower concentrations. In another series of experiments with the same type tumor, but with lower concentrations of the alkaloid, the range of mitotic activities varied from 78 divisions per 3000 cells to 380 metaphases. Further injection of the drug, then given in repeated equal doses, resulted in one half the number of mitotic figures obtained with twice the size of the dose. Du Bilier and Warren believe the optimal dose for this tumor is 0.1 mg./100 gm. body weight. The maximum effect is obtained in six hours with a single dose. With repeated doses, the effect becomes maximum at twelve hours. These unpredictable results led these authors to conclude that a trial of the effect of colchicine and X rays was infeasible.

Colchicine Combined with X Rays. Brues, Marble, and Jackson (1937) studied the combined effects of colchicine and radiation on normal and tumor tissue. Inbred strains of mice with tumor 180 and rats with the Walker tumor were used. The work attempted to standardize the effect on tumors and regenerating tissue of minute doses of colchicine administered daily. They observed a narrow range of dosage which caused metaphasic arrest yet did not kill the animals.

Radiation was administered in 10 daily doses of 300r, each beginning on the 11th day after the tumor was inoculated. Colchicine was administered 16 hours before irradiation began, presumably at the height of metaphase arrest. Regression occurred only in those animals that received massive doses of colchicine, which soon resulted in death of the animal. With smaller doses of colchicine used in the series, no tumor regressed.

Colchicine, Brues believes, has no effect on radiation response. Regression began no sooner when colchicine was given than when it was not given.

Large doses of colchicine may initiate tumor regression, but they are close to the lethal threshold and cause hemorrhage and metabolic changes in the tissue. Smaller doses of colchicine do not cause regression, nor do they affect the growth rate of the tumor.

Guyer and Claus (1939) investigated the influence exerted by X rays on colchicinized rats. They administered an aqueous solution of 0.1 mg. of colchicine/100 gm. of body weight and followed this with X rays (188r to 375r) administered in repeated doses. Biopsies were made from five rats at intervals of five hours and the mitotic number was determined in 3000 cells in comparable fields of the same tumor. The largest number of metaphases was obtained at 15 hours. At 45 hours, the number returned to 11 metaphases. These authors state, however, that other records showed an increase in mitotic count up to 72 hours.

In combination with X rays, the greatest lethal effect was noted over tissue treated with X rays alone, in which the cancerous rats were given 0.2 mg./100 gm. of body weight. Tissues previously colchicinized were removed and treated with 1500r. In these trials, the results showed that far greater lethal effects resulted from the combined use of the agents than from either of the agents used alone. These tissues, grafted on rats, failed to grow.

In experiments with tumors *in vivo*, the results given by colchicine and X rays were better than those obtained through either agent used alone.

More significant results were obtained only after some 20 series of experiments were performed, involving hundreds of mice. In these trials, one tumor, 16 days after implantation, attained the size of 1 to 1.5 cm., and a 0.1 mg./100 gms. body weight colchicine administration was followed 15 hours later by X rays (188r). This irradiation dose was given twice weekly. At the end of the sixth week, 15 animals of those that were colchicinized and X-rayed were healed. Four died. Of the group that only underwent irradiation, 11 were healed and three died. Eight of the animals treated with colchicine alone were healed, and ten were dead. In the following year, Guyer and Claus (1940) observed that colchicine, combined with distilled water, injected into the tumor, gave a larger number of recoveries. The cytoplasmic inclusions in these tumor cells were studied. The structure of the Golgi bodies and mitochondria was markedly changed within five hours after the injection of colchicine. The Golgi apparatus became broken up into fine droplets and the mitochondria took on the form of short rods or granules.

The combined effects of colchicine and X rays were studied by Hirshfeld, Tennant, and Oughterson (1940) on mammary carcinoma in mice. This procedure was expected to influence tumor growth more effectively than either of the agents used alone. The work of Hirshfeld *et al.* planned to determine the effect of colchicine at varying doses, to cause regression of tumors with colchicine in single or multiple doses, to test colchicine on spontaneous tumors, and, finally, to test the combined effects of colchicine and X rays. Albino mice, strain A, were used in these tests. Tumors treated with 0.0035 mg. gm. of body weight, produced toxic effects. The tumors were swollen, blue, hemorrhagic, and soft. The cells were in metaphase. Doses between 0.0009 and 0.0012 mg./gm. body weight produced the colchicine effect. The cells in division were in metaphase stage. Occasional telophase was observed, and no necrosis. Doses of 0.0016 mg./gm. of body weight injected at three- to five-day intervals retarded growth and prolonged life. Yet animals with spontaneous tumors injected with 0.001 mg. and killed at $9\frac{1}{2}$ hour intervals revealed no regression.

Mice with tumors 0.4 to 0.5 cm. in diameter were X-rayed with 2500r to 5000r eight to ten hours after a subcutaneous injection of a single dose of 0.0016 mg. of colchicine. This was followed by a second similar dose of colchicine and a second irradiation. A higher percentage of cures was obtained with colchicine combined with X rays (2500r) than with X rays alone. With the higher dosage of X rays (5000r), the results were similar to those where X rays were used alone. That colchicine does not strikingly increase the destructive effects of X rays was concluded. Yet a higher rate of curability than with X rays alone was recorded.

Seed, Slaughter, and Limarzi (1940) administered colchicine to patients with advanced cancer and, in some instances, irradiation preceded the colchicine treatment. The colchicine was administered in repeated doses of 2 mg. to 8 mg. and caused death, with leucopenia and marked diarrhea. Von Brücke and Von Hueber (1939) treated a patient with gastric carcinoma with metastatic nodules. They stressed the point that X rays must be

applied at the karyokinetic crises. Of two nodules treated, the one receiving colchicine and irradiation diminished in size and disappeared. On autopsy, the regressed nodule showed necrotic tissue with nonstainable nuclei. The nodule that was irradiated only remained unaffected by the treatment. The colchicine treatment consisted of eight injections of 0.1 mg. of the drug for nine days. Four irradiation treatments of 200r each were given.

Halberstaedter and Back (1943) studied the effect of colchicine and X rays on *Paramoecium caudatum* because of the simplicity of the material and the ease with which the effects on the individual cells could be observed. Three concentrations of colchicine were used. The protozoa were immersed in one solution for 48 hours, transferred to normal media, and irradiated. The lethal dose for paramoecia with prior colchicine treatment was approximately 50 per cent smaller than for those that were not previously colchicinized.

Ludford (1945), in summarizing the effects of colchicine combined with X rays, points out the need for determining the mitotic index by biopsy before and after treatments with X rays. Colchicine affects tumors that grow rapidly. He (Ludford) objects to colchicine because it acts on all tissue, especially blood-forming tissue and the C.N.S. No antidotes of colchicine are known. Complete regression of some animal tumors has been induced by the use of the drug far in excess of that required to arrest mitosis and just short of the minimum lethal dose. Regression of tumor growth occurs on the basis of vascular damage. Preliminary treatment with colchicine has not been found to increase significantly the sensitivity of tumors to radioactivity. Less toxic derivatives of colchicine require larger doses and fail to produce similar clinical results.

It should be mentioned in this connection that colchicine, although toxic, is still used in conditions of acute gout. The small doses necessary to effect relief apparently have no harmful influence on the body. The fact that colchicine operates at low levels of concentration precludes the need for using lethal doses. There is reason to believe that the possibilities of this drug in combination with X rays or other chemical or physical agents, in cancer, have not been exhausted. The tests made on animals have been inconclusive, although the work was difficult, trying, and extremely laborious. The type of cancer patients subjected to this therapeutic measure have been moribund, in terminal stage, and often post-operative. Even this type of case has been tried in very limited numbers.

An analysis of the evidence from animal experimentation presented above clearly indicates that relatively high, though sublethal, doses of colchicine, coupled with X rays, prolong the life of tumorous animals. Small doses of colchicine, coupled with the pure physical agent, gave inconclusive results, though the evidence in some cases showed that a better effect was produced than when either agent was used alone. Colchicine and X rays, applied to humans as a therapeutic measure in the treatment of cancer, have not been adequately explored. The available records show few cases studied, and these were haphazardly made on advanced stages of malignancy. It appears that the colchicine used in these cases was administered for its

toxic effects on the tumor. It may be said that no procedure for the study of cancer, with a view toward prolonging life and ameliorating the symptoms in the disease through a judicious use of colchicine, has so far been developed. Colchicine, the highly poisonous alkaloid, is used as a therapeutic agent in the treatment of man.

II. Cell Division in the Allium cepa

In this laboratory, a project dealing with the use of colchicine in the treatment of cancer was evolved as outlined above. The roots of *Allium cepa* were selected because of economy and adaptability and, finally, because of the ease with which the drug and X rays may be applied to a large number of specimens simultaneously. Also, the meristem of the root presents a simple fundamental tissue, uncomplicated by body fluids and their cells. The cytological effects of colchicine on the growing root tips of the common onion were known, and it was the purpose of this part of the project to determine the stage that gave the greatest number of arrested metaphases after treatment with the drug.

Resting bulbs of *Allium cepa*, var. Yellow Globe, were used exclusively. These were germinated by placing the root end of the bulb on top of a cylindrical vessel of 240 cc. capacity, filled with fresh cool water daily. The vessels were coated with an opaque substance to exclude light. After a period of seven to ten days, bulbs with a fair number of roots (more than 15) were transferred to similar vessels filled with 0.01 per cent aqueous solution of colchicine. To obtain as much variation in cell counts as possible, roots from four different bulbs were used in each test. The bulbs were divided into four series, each comprising 12 to 20 bulbs. The bulbs were examined at 6 hours, and thereafter at intervals of 3, 12, and 24 hours, up to 140 hours. After a given exposure, the root tips were carefully removed and the tissues were smeared and stained with aceto-carmine and chlorazol E. More than 4000 cells were counted for each exposure to colchicine, and the percentage of cells in the metaphase stage was calculated. Control bulbs were germinated in water at the same time, and the roots of these bulbs were studied too and smeared with the same stains.

Untreated Roots. The cells of four untreated roots were counted. Of 4112 cells, there were 3963 in the resting prophase stages and 149 in metaphase, 3.6 per cent of the total number of cells counted. (For graph and table, see Levine and Gelber, 1943.)

Colchicine-treated Roots. Roots exposed to 0.01 per cent colchicine for 6, 9, 12, 24, 36, 48, 72, 96, 120, and 140 hours were studied, and the number of metaphases among more than 4000 cells in four roots were counted. In the root tips exposed for 6 hours, 4112 cells were counted. A uniform number of metaphases, 71, 73, and 76 were scored, while the fourth root had 55. With this short exposure and low concentration of the drug, the number of metaphases doubled over the number in the untreated bulbs. In the 9-hour series, of the 4320 cells counted, 405 were in metaphase, 9.38 per cent of the total cells counted. Following this period, the tendency to an increase in the number of metaphases was now apparent, and this tendency became

obvious when the counts for the different periods of exposure were arranged chronologically. Among the roots exposed for 12 hours, the number of metaphases totaled 490 among 4195 cells counted (11.6 per cent). The number of metaphases in the 24-hour exposures reached a total of 637 among 4298 cells (14.88 per cent). An increase of 3.2 per cent above the number from the 12-hour group and an increase of 11.2 per cent of metaphases over the number counted in untreated roots were noted. The 24-hour exposure with 0.01 per cent solution of colchicine seemed to yield the maximum effect. It appeared that it represented the critical exposure for most roots, as a definite decrease in the number of metaphases was observed in the following treatments.

With the 36-hour treatment, and for longer periods, the number of metaphases dropped from 637 of 4298 cells, for the 24-hour period, to 423 of 4466 cells (9.4 per cent) at the 36th hour. In this series, the results indicated the prevalence of a toxic effect on the resting cells. Some of the nuclei completed their division phases and their reconstruction. Others gave evidence of degeneration.

In the succeeding exposures for longer periods, *i.e.*, 48, 72, 96, 120, and 140 hours, there was a gradual decline in the number of cells in metaphase until, in the last series of exposures, 140 hours, the number of metaphases fell below the number in the untreated roots. Of 4263 cells counted in root tips exposed for 140 hours, only 125 were in metaphase. Giant cells with abnormally shaped nuclei were seen. Telophase stages and cell divisions were not uncommon.

It appears from these studies that colchicine, in a given concentration for a given period up to and including 24 hours, increases the number of metaphases in the root tip of the onion by arresting the nucleus in that phase of division. The lack of an adequate spindle mechanism, however, does not prevent the chromosomes from dividing, but apparently its failure to function prevents cell division and gives rise to giant cells with multiploid chromosome numbers. The cell colchicinized by longer periods of exposure than 24 hours interferes with the resting and prophase stages. It seems that cells go into the division phase less frequently. It is clear that this stage begins with the 24-hour exposure. The extreme variation in the number of metaphases counted in the four roots at the 24th hour indicated the beginning of the toxic influence exerted by colchicine or the inability of the colchicine to hold the dividing nucleus in metaphase any longer. These observations lead to the conclusion that the number of metaphases in the colchicinized root tip is predictable within the limits of variation of the 24-hour treatment as applied in these studies. It indicated that simple plant tissues like the onion root tip are suitable for the initial studies of the combined effects of colchicine and X rays.

III. Colchicine and X Rays

Root tips of *Allium cepa* were subjected to various concentrations of colchicine by Havas (1937), Levan (1938), Kostoff (1938), Shimamura (1939), and others. In this laboratory, in collaboration with Dr. Joseph

FIGURE 1. Bulbs germinated for 12 days in tap water, followed by a 0.01 per cent aqueous solution of colchicine; bulb 11–24 hours, 16–48 hours, 28–135 hours, 29-water control. The exposures were made in series of 6 to 40 bulbs. Note the hypertrophied root tips. × ¼.

FIGURE 2. Bulbs germinated in tap water for three days followed by exposure to 0.01 per cent colchicine for 48 hours. Eleven days after the return to water, the colchicine hypertrophies persist but the root tips resume growth. × ¼.

Lein (Levine and Lein, 1941), it was shown that a concentration of 0.001 per cent was sufficient to arrest growth of the root which is under the influence of the drug. With a tenfold increase of the concentration, 0.01 per cent, not only was the arrest marked, but the region behind the meristem and including its proximate portion began to show evidence of hypertrophy at the 18- to 24-hour period and reached its full development at the 48th hour of treatment, when it measured 1–2 mm. This hypertrophy was first described by Havas (1937) as "phytocarcinoma" and referred to by Levan (1938) as "colchicine tumor." Continued exposure to colchicine beyond the 48th hour had little or no effect on these hypertrophied root tips. The return to water of these colchicinized roots after more than 200 hours of treatment caused these roots to assume growth in three to four days. The growth started at the distal portion of the hypertrophy and continued under favorable conditions. This concentration was used throughout the experiments. These hypertrophies served as indicators for the efficacy of the irradiation treatment. The roots, irradiated only, or kept in water as controls, were measured before and at various periods after treatment. Microscopic examination of roots, immersed in aqueous solution of colchicine at this concentration for as long as 200 hours, revealed only a reduction in the size of the meristematic zone upon return to water. The new growth of root tissue was only smaller in diameter.

In a series of six experiments, the effect of a single dose of X rays of 900r on colchicinized root tips on bulbs of the Yellow Globe variety of *Allium cepa* were studied.* One hundred and thirty bulbs were grown in fresh tap water from 3 to 12 days. Eighty-two were then colchicinized for 24, 48, 72, 120, 135, and 140 hours. Thirty-two others were irradiated; of this number, 20 were colchicinized. There were three or four irradiated colchicinized bulbs in each of the six experiments. The controls, 98 in number, consisted of non-X-rayed colchicinized bulbs and others simply grown in water (FIGURES 1–4).

Of the bulbs selected for colchicine and irradiation, 12 were exposed to 0.01 per cent colchicine solution for 120 hours. Five bulbs were irradiated with 900r and returned to water. The gross differences between the colchicine X-rayed root tips (C-X) and those treated only with colchicine (C) were studied. All the C-X group appeared viable but for the presence of an occasional flaccid root. Yet no outgrowth from any of the roots occurred. Eight days after their return to water the C-controls produced extensive outgrowth from the bulbous hypertrophies, showing no permanent impairment or destruction of the meristem.

Root tips exposed to C for 72 hours following a similar irradiation, and

* The X-ray machine used delivered 95–133 roentgens (r) per minute during the different exposures made. The voltage was 200 KV, 25MA, with filters of 0.5 mm. copper and 3 mm. aluminum. The distance from the target varied for a given set of onions from 42 to 67 cm. The actual exposure varied from 11 ¼ to 30 minutes. The time consumed by the irradiation varied from 17 to 40 minutes. The portal of the X-ray machine was open and the field of irradiation was considered uniformly covered. The non-X-rayed bulbs in a given experiment were removed from the cylinders for a period consumed by irradiation. Precautions were taken to avoid drying of roots during these procedures. After treatment, roots were fixed or returned to water and then fixed for microscopic examination at given intervals. Bouin's, Carnoy's, or Flemming's weaker solution was used and the tissues were stained with Flemming's triple stain or Heidenhain's iron-haematoxylin. Other short, though efficient, staining methods were employed. The above techniques were used throughout the study.

FIGURE 3. Bulbs germinated in tap water for three days and colchicinized for 48 hours followed by exposure to X rays (900r) Photograph made 11 days after the bulbs were returned to water. Growth persisted for four to five days. × ½.

FIGURE 4. Samples of bulbs colchicinized for 48 hours and X-rayed: bulb 2—1500r; 7—3000r; bulb 5, noncolchicinized—1500r; 13—3000r; bulb 18 colchicine only; bulb 10 in water only; photograph made 12 days after colchicine and X rays. × ½.

then returned to water, showed no evidence of their ability to resume growth. The root tips of bulbs colchicinized only, resumed growth in three to four

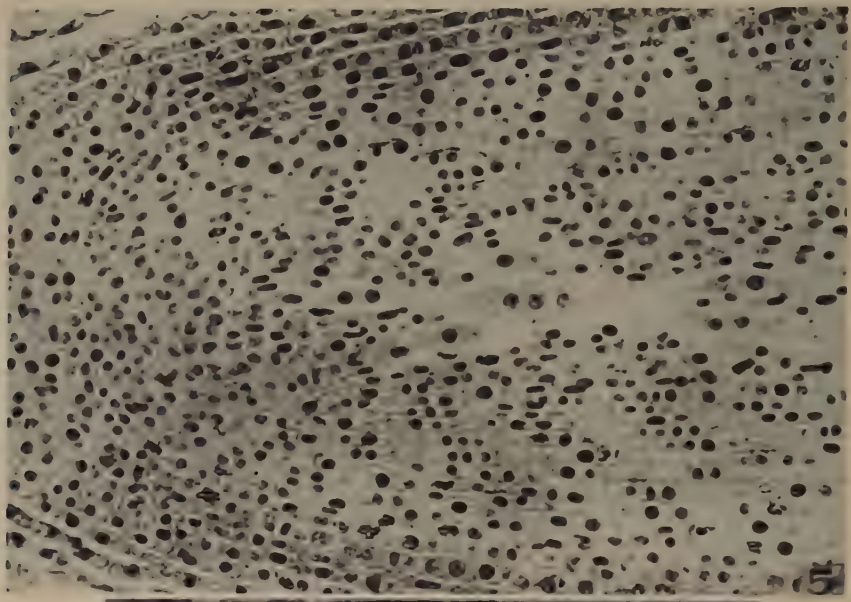
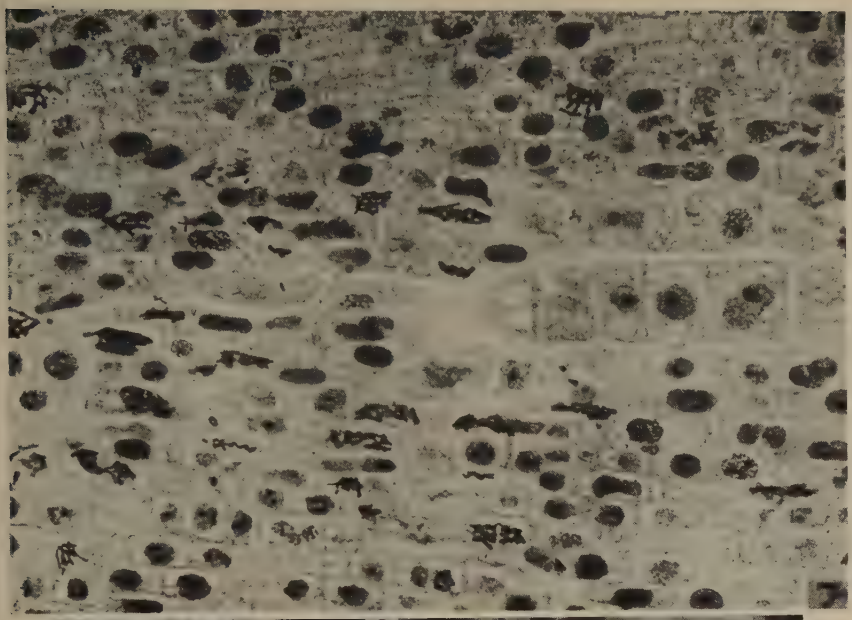


FIGURE 5. Longitudinal section of a root tip exposed to colchicine for six hours. Note metaphase arrest. × 250.

FIGURE 6. Selected area from FIGURE 5 showing separation of the chromatids with evidence of stickiness; incomplete separation. X-shaped chromosomes are not infrequent. × 1800.

days after their return to water. Of 24 onions treated with C at the same concentration for 48 hours and similarly irradiated, growth was resumed when they were returned to water, 11½ days after the combined treatment.



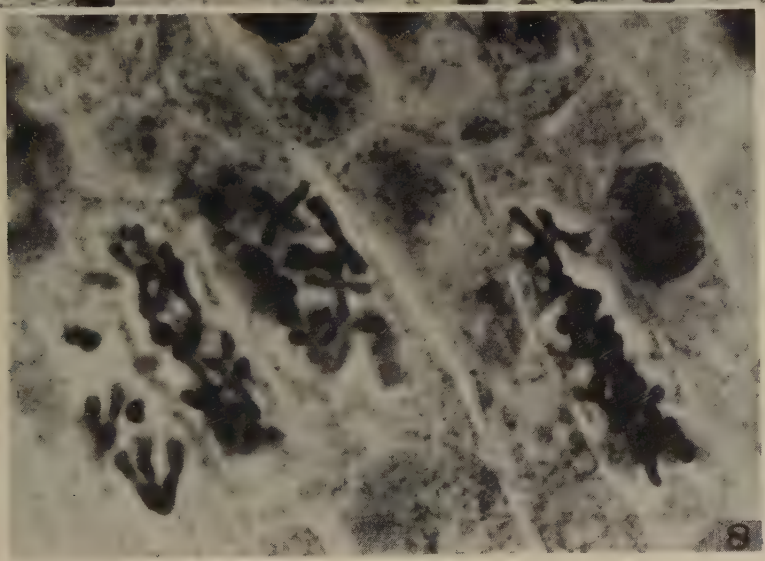


FIGURE 7. Section of root tip showing a large number of cells in metaphase 18 hours after colchicine. Note the circle and X-shaped chromosomes. × 480

FIGURE 8. Cells from a similarly treated root tip as in FIGURE 7. Spiral arrangements of the chromosomes are seen; chromosomes shortened and compactly arranged. × 1800.

These C-X bulbs showed root extension of approximately 0.5 cm. Daily measurements of the length of the roots, however, showed the growth to be slow and suggested an elongation process rather than one of cellular

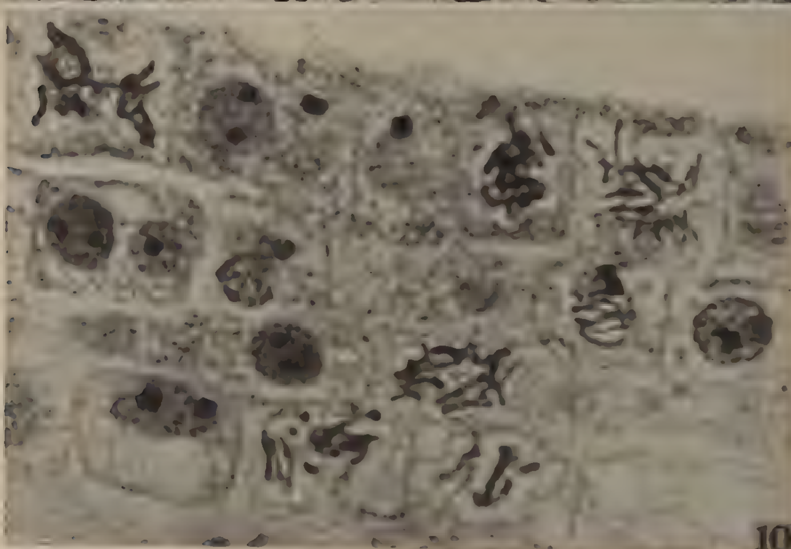


FIGURE 9. Root tip exposed to colchicine for 24 hours. × 500.
FIGURE 10. Portion of a root tip showing separation of chromatids. Binucleate cells. Separation of chromosomes into two unequal masses. × 1060.

proliferation. Continued growth failed after the sixth or seventh day. C' controls showed resumption of growth on return to water. The significance of these results made it necessary to test further the effect of the irradiation on 48-hour C' root tips. In this test, 30 bulbs were used. The

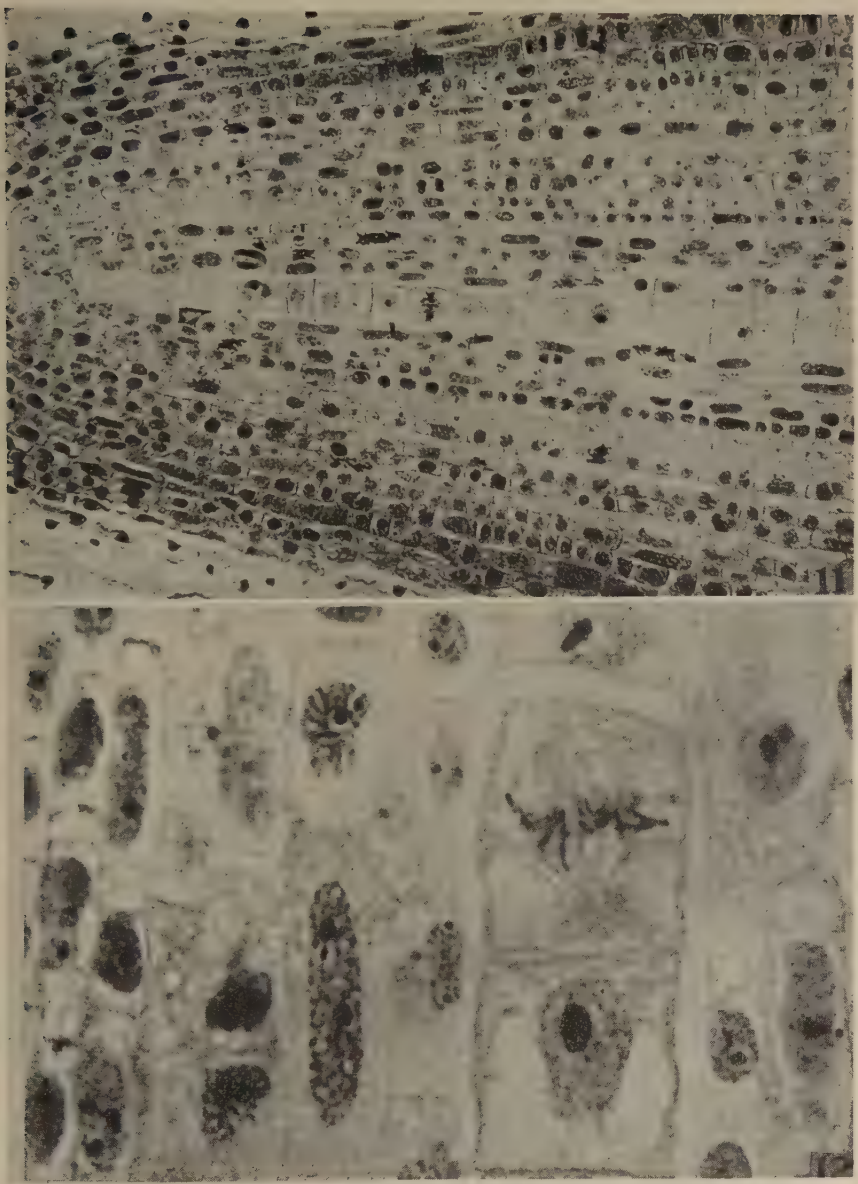


FIGURE 11. Median section of a root tip exposed to the alkaloid for 36 hours, returned to water for 24 hours before fixation. × 240.

FIGURE 12. A portion of an adjacent section shown in FIGURE 11; normal division with spindle figure in pleurome cell. × 1060.

results confirmed the findings observed above. The control non-C bulbs that were kept in water for nine days and which were X-rayed simultaneously with the C-bulbs showed little effect after their return to water. The roots

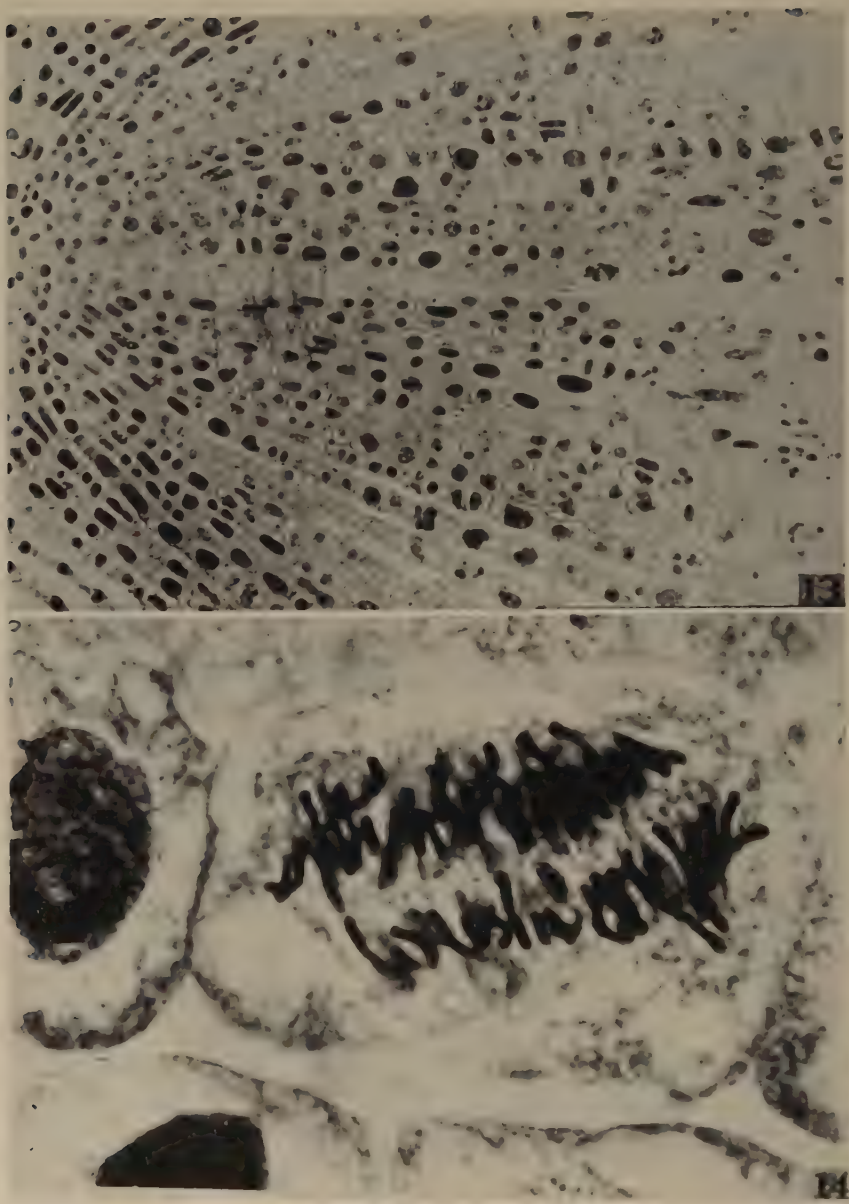
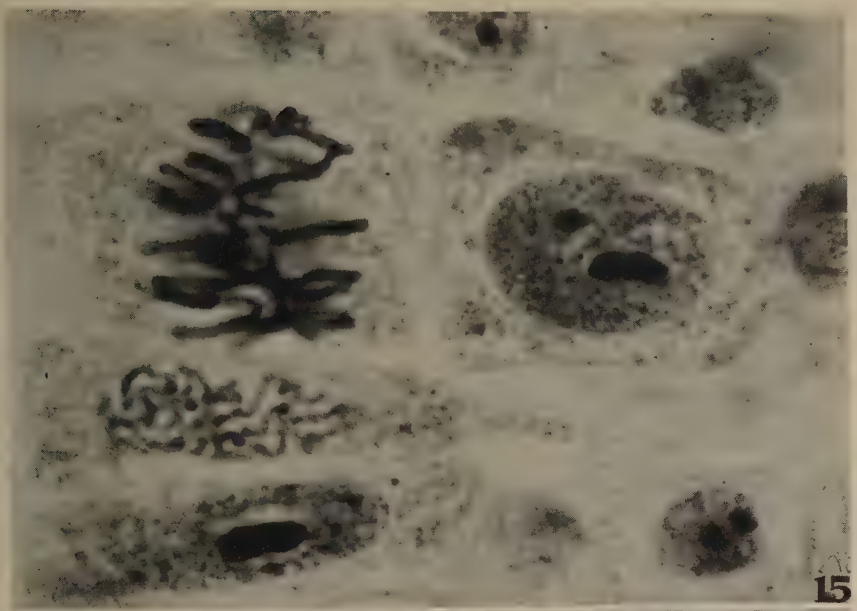


FIGURE 13. Section of root tip in colchicine for 48 hours, fixed 24 hours after water. Note hypertrophied cells with lobed nuclei. × 250.

FIGURE 14. Giant cell from section of root tip similarly treated as in FIGURE 13; note large number of chromosomes in anaphase stage. × 1800.



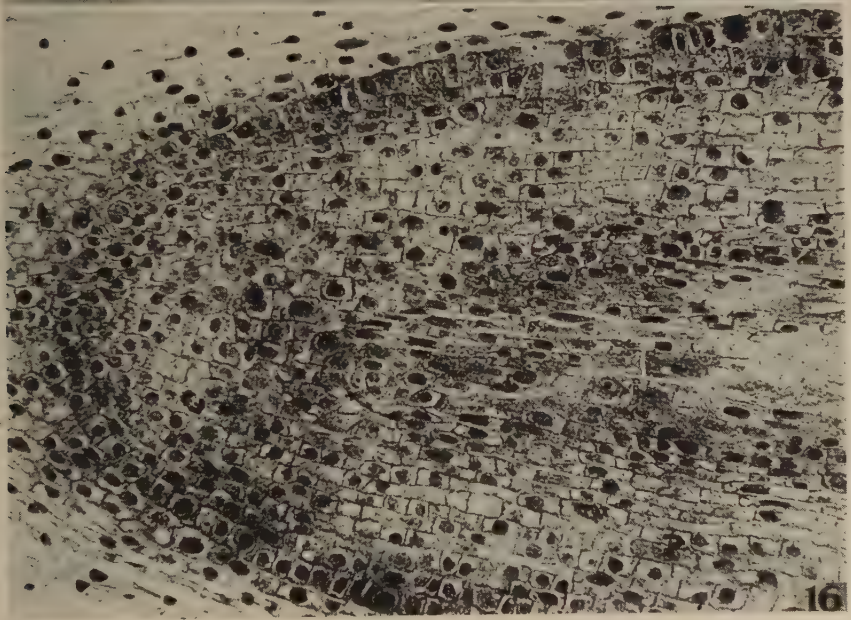


FIGURE 15. Another collection of cells from similarly treated root tip showing various phases in division. × 1800

FIGURE 16. Root tip exposed to colchicine for 125 hours followed by water for 90 hours. Normal division reappears. × 240.

appeared active, the tips were normal, and there was only a slight temporary arrest of growth.

FIGURE 17. Portion of a root tip after exposure to colchicine for 200 hours; note region of injury, few division figures occur. × 500.

FIGURE 18. Part of a root tip exposed to colchicine for 24 hours and X rays (900r), fixed two hours after irradiation. × 480.

Root tips exposed to C for 24 hours and irradiated with 900r grew after they were returned to water. Those that were colchicinized for 135 140

hours and irradiated reacted like those that were irradiated after 120 hours of C.

Irradiation with 900r has a temporary retarding effect on the growth of the onion root tips, but, when it is preceded by an exposure to C for more than 48 hours, complete inhibition of growth is attained without causing immediate death of the tissue. Root tips exposed to C for 48 hours, followed by 900r, became impaired in growth with complete exhaustion of growth five to seven days after their return to normal conditions.

Cytological Changes Induced by C-X. Colchicinized and X-rayed roots were selected at random for microscopic examination from all the bulbs subjected to these agents. The untreated control root tips were kept in water for 15 days and showed normal cellular structure with a moderate number of nuclear and cell divisions. Bulbs treated with C for 6, 18, and 24 hours presented the C-induced metaphases (FIGURES 5–10). In the root cap cells of these roots, the nuclei were homogeneously stained with Flemming's triple stain. The dermatogen cells had metaphase stages and occasional late telophases with normal spindles. In these metaphases, some chromosomes were fused, while others were distinct and showed evidence of longitudinal splitting or separation, with some residual points of adhesion (stickiness). Spiralization of the chromosomes was commonly observed in the prophase stages; ring and typical X-shaped chromosomes appeared. The nuclei in the resting stages were well differentiated and the chromatin was characteristically stained with the gentian violet according to Flemming's method (FIGURES 11–17). The granular material in the cytoplasm seemed to be concentrated, forming a loose reticulum. There was no evidence of nuclear or cytoplasmic injury.

Colchicine and Irradiation of 900r

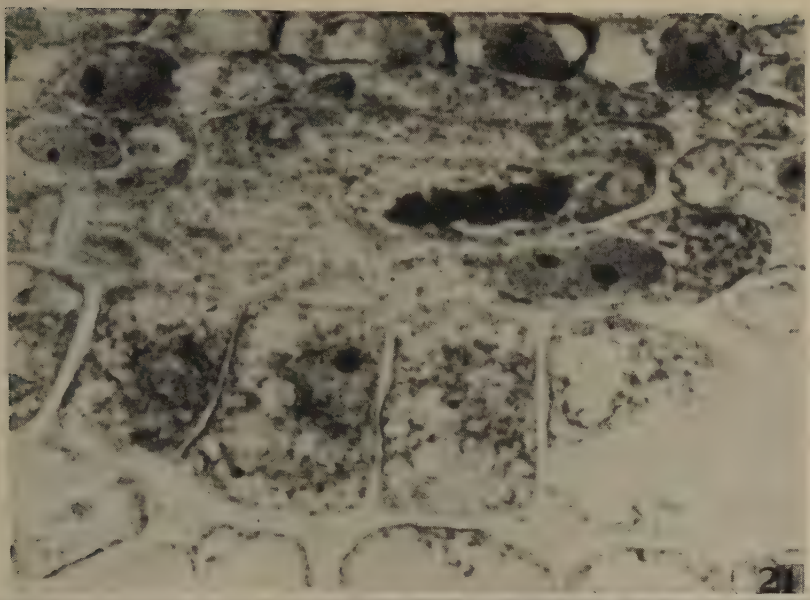
In sections of root tips colchicinized for 24 hours and irradiated with 900r, and fixed two hours after their return to water, marked clumping of the chromosomes was noted in the metaphases (FIGURES 18, 19). A number of prophases appeared in these preparations, which were apparently normal. A detailed study of the cells in division showed pairs of chromatids in parallel arrangement. Others were partially split, forming "X" figures and rings. The X-ray control plants, non-colchicinized in this series, showed some metaphases and less marked chromosomal aberrations than those treated with C and X, yet split chromosomes were found in tissues fixed two hours after treatment. Roots in this series of C and X, examined eight days after their return to water, had active viable cells. Roots exposed to colchicine for 48 hours and then followed by X rays of 900r were compared with those that were colchicinized only or X-rayed only. All were returned to water and studied microscopically at various intervals after treatment.

In the root tips colchicinized for 48 hours, the chromosomes in the metaphases are fused and contracted and fragmentation occurs. These observations are in accord with Levan (1936), Shimamura (1939), Berger and Witkus (1943), and others. There appeared to be a number of masses of gentian violet staining material in some cells. The chromosomes in these prepara-

FIGURE 19. Root tip colchicinized for 24 hours followed by X rays (900r), fixed 8 days after return to water. Note cells are viable, divisions few. × 250.

FIGURE 20. Portion of a root tip exposed to colchicine for 48 hours and 900r, fixed one hour and a half afterward. × 480.

tions frequently appeared indistinguishable from those subjected to X and suggest the radiomimetic effect described by Dustin. The resting nuclei were densely stained, while the cytoplasm showed an intensely staining granular structure. Vacuoles were present. Sections of root tips from the



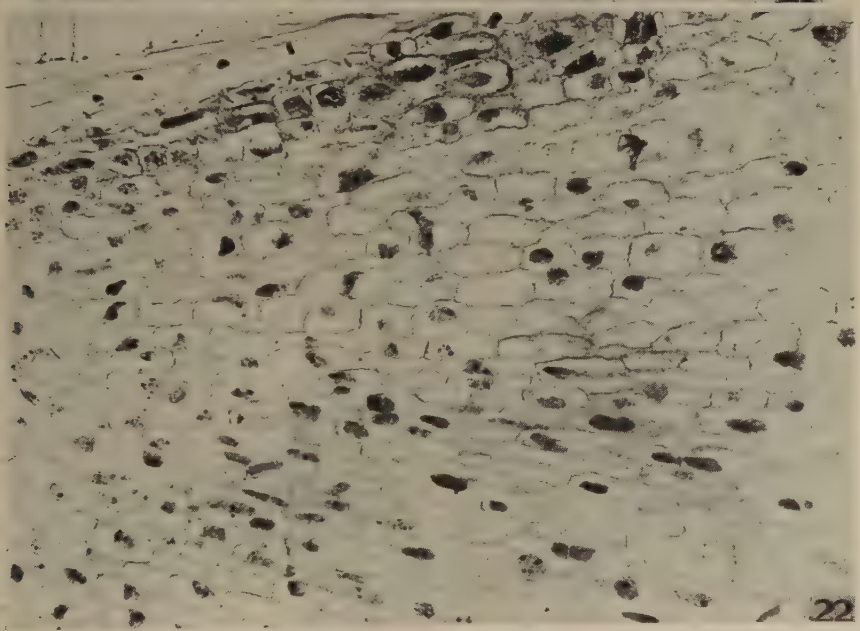
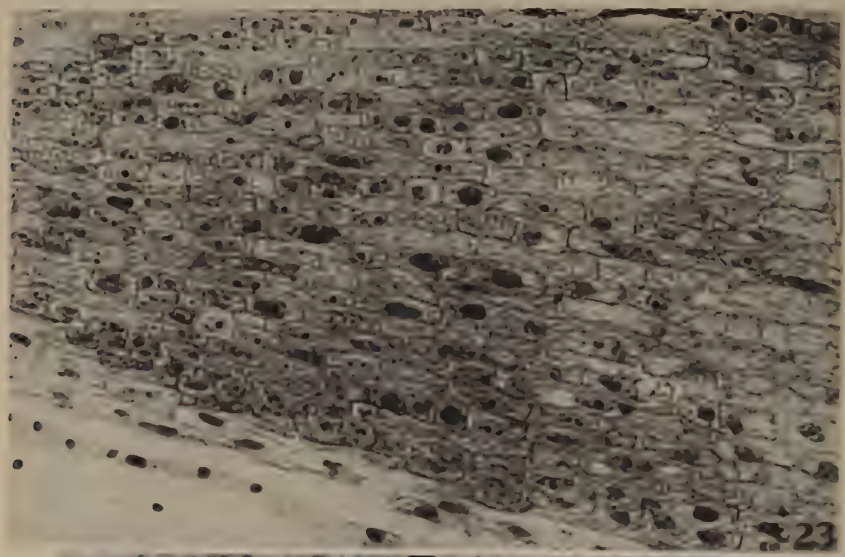


FIGURE 21. Part of root as in FIGURE 20, shows clumping of chromosome mass. × 1060.
FIGURE 22. Section of root tip exposed to colchicine for 48 hours and 900r, returned to water for 48 hours. Note the large nuclei with several nucleoli. × 250

same bulb made 24 hours after the C treatment showed normal telophases, yet deeply stained nuclei and possibly dead cells occurred. At 72 hours after the treatment, various normal mitotic stages reappeared.

Root tips colchicinized for 44–48 hours and irradiated with 900r, studied one hour and 35 minutes after their return to water, showed dividing nuclei.



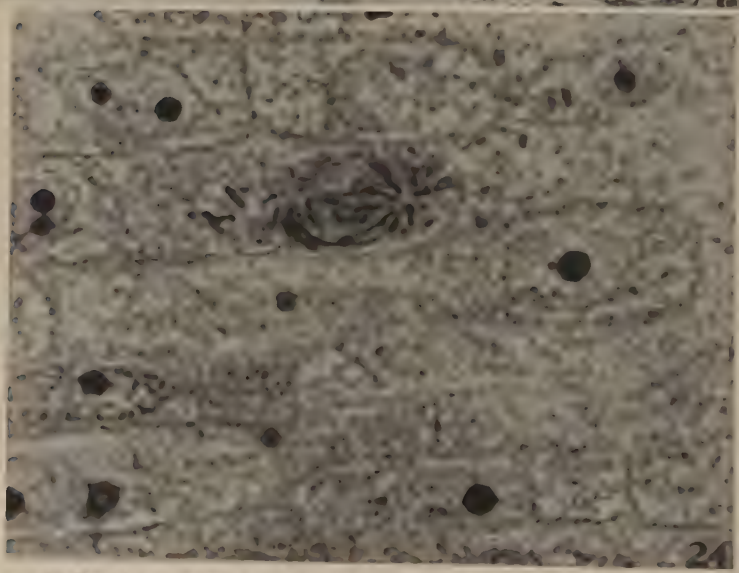


FIGURE 23. Root tips exposed to colchicine for 48 hours and irradiated with 900r, fixed 95 hours after treatment. Note normal divisi n stages. × 240.

FIGURE 24. Portion of FIGURE 23 under higher magnification. × 1060.

and revealed elongated chromosomes (FIGURES 20, 21). The resting nuclei were lobed in a highly vacuolated cytoplasm. In these cells, the cytoplasm appeared to be reticulate, with densely staining granules lying in the mesh-

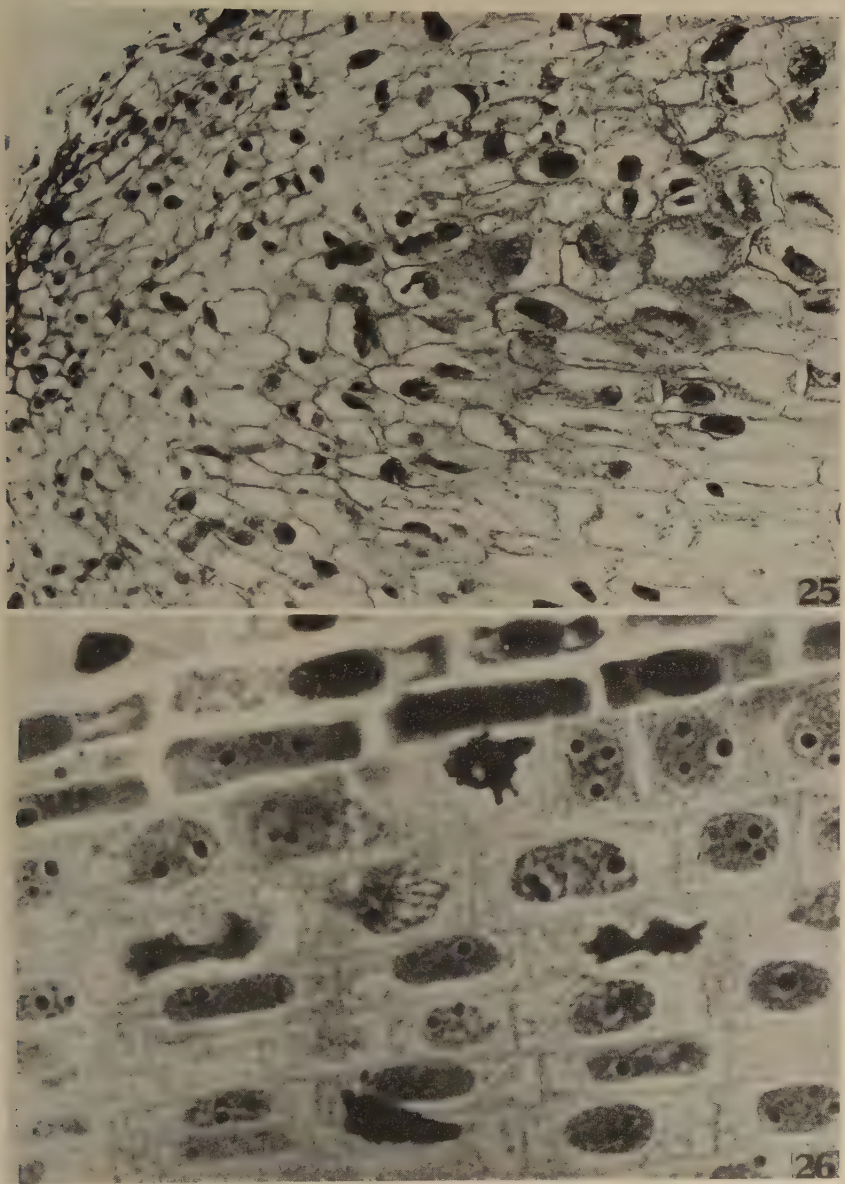


FIGURE 25. Root tip exposed to colchicine for 125 hours and 900r, fixed 90 hours after treatment; note aberrant cells, division figures few, polyploid. × 250.

FIGURE 26. Section of root tip exposed to colchicine for 18 hours and 1500r, note the clumping chromosome. × 1060.

work. Forty-eight hours after C-X treatment, root tips show slightly elongated hypertrophied cells, although they appear to be arranged in regular tiers. The nuclei were irregular in shape and stained homogeneously. In

preparations made 80 hours after the treatment, recovery had set in. Normal nuclear divisions were in progress and the intense cytoplasmic vacuolization had disappeared. There is evidence in these preparations that the chromosome number increased, but no counts were made. The outgrowths from the C-X hypertrophied tips were thin, and division stages were not abundant. The active cells were stained like normal meristematic tissue (FIGURES 22–24).

Root tips, exposed to colchicine 72, 125, and 140 hours, produced new outgrowths, which grew uninterruptedly from the bulbous tips. Aberrant cells in the hypertrophied part of the root have giant nuclei. Normal cells with a small number of the nuclei in division were seen. C-X-treated roots, examined 96 hours afterward, fail to grow on return to water. The cells are large and vacuolate and have homogeneously stained nuclei. The outer margin of cells (dermatogen) shows evidence of injury. Growth in this tissue was not followed beyond the fifth day (FIGURES 25, 26).

Root tips exposed to C for 125 and 140 hours and treated with X showed no root caps. The cytoplasm was strikingly fibrillar. Evidence of giant cells was present and a number of cells appeared necrotic. Spaces filled by densely staining material gave evidence of destruction and displacement. These roots failed to resume growth when returned to water after the C-X treatment. The colchicinized root tips irradiated with 900r showed that the length of the C exposure influenced recovery.

Long exposures of 72–140 hours, followed by X, completely inhibit growth, although the roots are not destroyed. Shorter C exposures (48 hours and less) inhibit growth temporarily, for the bulbous tips produce outgrowths. Obviously, the inhibiting influence is not the function of the irradiation (900r), nor the colchicine alone, which has only a temporarily retarding effect, but the combined influence of these agents. The short colchicine exposure, followed by irradiation, left unimpaired meristematic cells which are capable of producing new growth in the usual span of time. The long colchicine exposure, coupled with 900r, inhibited and impaired the fundamental tissues and no outgrowth occurred from the hypertrophied root tips. Neither irradiated (900r) nor colchicinized root tips fail to show the gross and microscopic changes induced by the application of both agents.

Colchicine Combined with 1500r or 3000r

In addition to the "Yellow Globe" variety culled from the open market, the Brigham strain of "Yellow Globe" variety was used in these experiments. The onions used were carefully selected and then chilled for 12 to 14 days before they were set to germination in water cultures. After a period of a week, each bulb had developed an abundant number of roots for study (FIGURES 27–31).

In one experiment, 18 bulbs of the Brigham "Yellow Globe" variety were used. The specimens weighed from 58 to 94 oz. and germinated uniformly eight days after they were placed in water. These were divided into two groups and six bulbs of each were placed in a 0.01 per cent aqueous solution of colchicine, while three of each group remained in water. After an ex-

posure to C for 48 hours, five bulbs were irradiated with 1500r. Two bulbs not colchicinized were irradiated only. In the other set, four bulbs were

FIGURE 27. Median section of a root tip exposed to colchicine for 36 hours and 1500r. × 280.
FIGURE 28. Portion of root tip shown in FIGURE 27. × 1060.

colchicinized for a similar period and exposed to 3000r. Two non-colchicinized bulbs were included in this exposure. The roots of two colchicinized and two non-treated bulbs served as controls. The root tips of these bulbs

were studied at various intervals after the treatment. The C treated bulbs, irradiated with 1500r or 3000r, showed clearly the complete inhibition of growth of the bulbous tips nine days after their return to water. The non-C

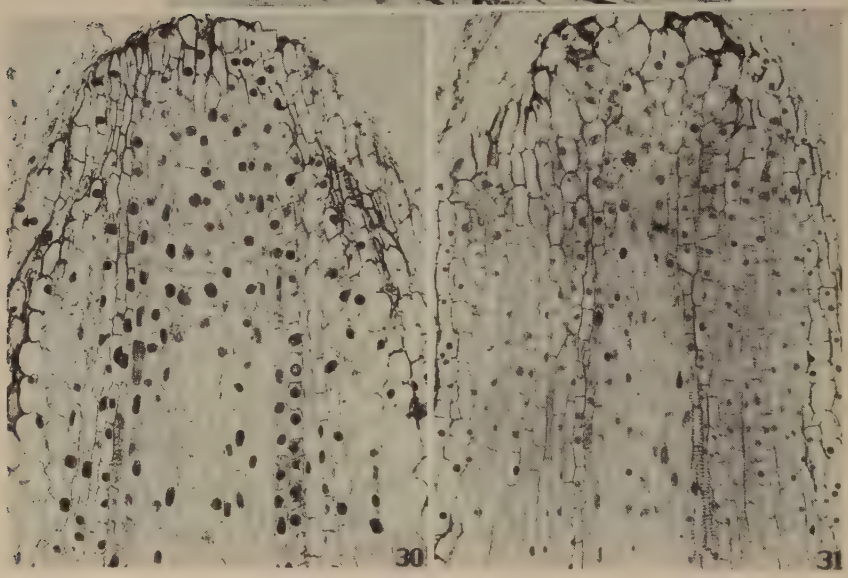


FIGURE 29. Root tip exposed to colchicine for 48 hours followed by irradiation with 1500r; fixed 120 hours after X rays. Note viable cells. × 250.

FIGURE 30. Section of a root tip exposed to colchicine for 48 hours and 3000r, fixed 120 hours after X rays. Note injured cells—no root cap. × 250.

FIGURE 31. Section of root tip irradiated only with 3000r, fixed 13 days after treatment, compare with FIGURE 30. × 250.

bulbs, irradiated with 3000r, showed some root injury. The roots irradiated with 1500r or 3000r only showed characteristic twisting of the tips, indicating extensive though irregular cell impairment. The C bulbs resumed growth of the root tips four days after their return to water. Measurements of the

water control root revealed daily increment of 2 to 5 mm. among the shorter younger roots. There were few flaccid roots after being in water for 29 days. The primary roots had produced secondaries.

The roots from some bulbs selected at random from each of these series were counted and their length measured before and after their respective treatments. The roots were removed from the bulbs 19 days after their return to water, in order to study to a greater advantage the gross effects produced by the treatments. The roots irradiated with 1500r only had been in water for ten days prior to their irradiation and 19 days afterward. The X rays' effects seemed to be confined to the root tips, where slight twisting and bending were observed, as noted above. There was evidence of growth but no secondary roots appeared. The 3000r-treated root tips showed more marked distortion of the tip region of the long roots. A number of roots in this group were flaccid. The root tips appeared white and active and capable of growth.

The roots that were colchicinized only resumed growth. Many secondary roots were formed on these outgrowths, while a comparatively large number of new roots had developed from the bulbs during their post-colchicine period in water.

The C and X roots (1500r), 19 days after treatment, showed a very limited extension of the bulbous tip in only a few instances. The inhibition of growth appeared to be nearly complete. Similar gross effects were observed with C exposure for 72 hours and X with 900r. C root tips treated with 3000r in this series showed complete inactivity of all bulbous tips 19 days after treatment. No new roots were formed from the bulbs tested. It seems from these experiments that complete inhibition of growth of root tips exposed to 0.01 per cent solution of C for 48 hours may be attained with an X-ray dose of a little over 1500r. It appears further that the 48-hour C bulbs are more vulnerable to X treatment, even though the time of exposure occurred when the number of dividing cells had passed the peak of metaphase arrest. The maximum toxic influence of the drug is attained, at the concentration used, at approximately 24 hours of exposure, since prolonged C treatment up to 200 hours failed to inhibit subsequent growth. The hypertrophies induced by the colchicine furnished a ready means of demonstrating growth phenomena in the root and showed clearly the inhibition effects after the two agents, C and X, are employed. Microscopic studies of these roots, together with daily measurement, furnished further evidence on this point.

Cytological Changes Induced with 1500r and 3000r

The root tips of the "Brigham Yellow Globe" variety of *Allium cepa* were studied microscopically at various periods after exposure to colchicine. The root tips fixed in this series, from samplings taken from all the bulbs before treatment, showed an average karyokinetic activity. Bulbs exposed to colchicine for six hours have an accumulation of metaphases, in which the chromosome already showed unspiraling and separation of the chromatids to form X's and rings. Exposures for 12, 18, 24, and 48 hours reveal an

increasing number of metaphases, except in the 48-hour period. Root tips of the variety of onion, colchicinized for 48 hours and examined 24 hours after their return to water, recovered from metaphase stages. Giant cells with lobulate nuclei and normal nuclear and cell division were found. Though the hypertrophies of the root tips were marked, the cells were definitely oriented in regular tiers. The cell arrangements were so regular that no analogy between this hypertrophy and the crown gall disease of plants or malignant growths of animals could be established. The "colchicine tumor" (Levan, 1938), or the colchicine phytocarcinoma (Havas, 1937), does not fall into any category of tumor growth.

Blakeslee (1937), Levan (1938), and others showed that polyploidy occurs after colchicine treatment. Levan counted large numbers of chromosomes in the colchicinized root tip. Increasingly large masses of distinct, clearly differentiated, apparently normal chromosomes were observed in the cells of the same root tip in my preparations. Polyploidy has been frequently observed in the C root tips, in crown gall, and in cancer of the fish, mouse, rat, and man (Levine, 1931, 1948). Polyploidy does not seem to be a criterion for malignancy. It is of interest to note that mitotic poisons, X rays and radium, low and high temperatures, and the responses of the plant and animal cell to yet unknown agents in cancer are associated with mitotic disturbances which result in micronuclei, giant nuclei, and polyploidy. The precursors to these stages may be found in the state of the cytoplasm and its change in its physical as well as chemical state, as indicated by Lewis (1948), Chambers (1948), Chalkley (1948), and others in this monograph. The separation of the chromosome into chromatids may be interpreted (Östergren, 1943) as being due to the physical property of the chromosomes. The final separation of the chromatids to form the late anaphase or telophase stages without the intervention of spindle fibers suggests a gelation of the cytoplasm, with contractility, which causes the imbedded chromatids to move to opposite poles of the cell. Causes for invagination and division of the treated animal cells are not clear. In the plant cells shown in these preparations, a granular material is found, between the two masses of chromosomes, which is finally developed into a cell wall. When the gelation process does not ensue, tetraploid, octoploid, and polyploid cells form the residuum of the mitotic process.

Non-colchicinized Irradiated Roots. Normal root tips of the onion, irradiated with 1500r or 3000r, have contracted nuclei apparently resting, or permanently impaired. The first 24 hours after irradiation show no division figures. Several days later, however, normal division stages appear. Roots irradiated with 3000r and examined 24 hours later are free of division figures. The nuclei are irregular in outline and appear as densely stained undifferentiated bodies. The inner layers of the roots still retain well-differentiated cytoplasmic and nuclear structures. After C and X 3000r, the inner layers of cells are heavily stained. No division figures appear. The peripheral cells of the root show marked contraction, dense nuclear staining, and necrosis.

This study shows clearly that the combined effects of C and X are more

effective in reducing the rate of growth and bringing about necrosis than X rays alone.

Effect of Short Exposures to Colchicine

The fact that, in the onion root tips exposed to colchicine for more than 48 hours and irradiation of 900r or 1500r, retarded and inhibited root tip growth made it necessary to study the effects of irradiation on roots exposed to this alkaloid for periods shorter than, and between, 24 and 48 hours. Eighteen onions of the "Yellow Globe" variety were exposed to 0.01 per cent aqueous solution of colchicine after germination for eight days. The colchicinization was for either 18 or 36 hours and this was followed by irradiation with 1500r. Some onions remained untreated, while others were irradiated only, and a fourth group was only colchicinized.

Root tips that were irradiated only showed no development other than that recorded above. They resume growth after a period of three to four days in water. The C and X roots, 18 hours and 1500r, were not permanently affected for 23 days after the treatment. They showed slight increases in the length of roots as compared with the water controls. Secondary roots were formed in the distal portion of some of the long primary roots and some new roots had developed. The colchicine, at 18-hour exposure, combined with 1500r, appeared to produce no marked observable changes. Microscopically, the C roots fixed after 18 hours had numerous metaphase stages. Twenty-four hours after C, root tips have normal cells, with nuclei in the prophase and telophase stages. The C and X roots, however, fixed after irradiation, showed intensified clumping of chromosomes, fragmentation and partial fusion, "stickiness," or incomplete separation of the chromatids. Spindle figures were conspicuously absent.

While colchicine causes analogous cytological changes at 6, 12, 18, 24, and 48 hours, the larger exposures induce some microscopically unrecognizable alterations. This permits the cell to resume growth after the shorter exposures to C and 1500r, but arrests growth permanently and completely with larger exposure to C with this dosage of irradiation, as shown above. This is further evidence for the fact that the irradiation is not the sole factor that inhibits growth, but that both agents supplement each other.

Bulbs exposed to colchicine for 36 hours and then followed by irradiation with 1500r showed more intensified reaction than that reported above for 24 hours with similar irradiation. The hypertrophied root tips of the C-X series grew slightly after 8 days as compared with the irradiated, colchicinized, or water controls. Photographic records made it possible to contrast these series at uniform intervals of time. Twenty-two days after the C and X treatment, there appeared to be little change in the length of the C-X roots. There were some flaccid, water-soaked, and healthy roots with bulbous tips with small outgrowths from them. No new roots were formed. The C-X effects after 36 hours and 1500r show slight difference in comparison to the 24-hour 1500r and 48-hour C-1500r treatment. The complete inhibition of growth is not attained with the combination of colchicine exposure for 36 hours and irradiation of 1500r.

It appears that the optimum effect is reached when irradiations of 1500r are combined with an exposure to colchicine of more than 36 hours. The combination of 900r with 0.01 per cent aqueous solution of colchicine for more than 48 hours, or less than 72 hours, or 1500r with 48 hours exposure to this same concentration of colchicine, is most effective in arresting root growth of the onion root tip—a fundamental tissue with more or less uniform embryonic vegetative cells.

It appears that the longer C exposure induced some non-recognizable toxic effects in the cell which sensitize it to the irradiation. The fact that the maximum X-ray effect is induced after exposure to colchicine for more than 48 hours with 900r, and less than 72 hours with 1500r, indicates that the action of the X is independent of the nuclear division phase but is in some way dependent upon the influence exerted by the colchicine.

IV. Colchicine and the Mechanics of Cell Division in Human Cancer

Cancer patients suffering from gout gave the first clue to the possible value of this alkaloid in malignancy. Dominici (1932) and, later, Amoroso (1935) found, as is indicated above, not only that this drug, given in small doses, alleviated the pains in this acute arthritic condition, but that improvement and arrest of the cancerous condition was noted. This observation led Amoroso to investigate the use of colchicine in the growth of tumors in mice mentioned above.

The use of X rays in combination with colchicine, though developed several years later, appeared to be undertaken as a means of last resort and only two reports on such attempts appear in the available literature. In one account, a single patient with an advanced gastric carcinoma with metastatic nodules in the skin was described. These nodules were treated, one with colchicine and X rays, and the other with colchicine alone. Brücke and Heuber (1939) pointed out the need of establishing the karyokinetic crisis induced by colchicine before radiation was applied, but no record of this type of examination is reported by them. Seed, Slaughter, and Limarzi (1940) used irradiation before and after administration of large doses of colchicine.

Oughterson, Tennant, and Hershfeld (1937) had undertaken to establish the karyokinetic crisis in human cancer tissue after injections of colchicine, but carried their examination to biopsy only 12 hours after the injection of the drug. Their observations show an increase in metaphases from the control average of 2:6 in oil immersion fields (O.I.F.) to 19.6 metaphases (O.I.F.) 12 hours after colchicine injection. They concluded that colchicine makes it possible in some instances to obtain a more accurate index of the rate of growth of a tumor.

The following deals with the direct approach to the application of colchicine and X rays in the treatment of human cancer. It is concerned with the establishment of an approximate period after colchicine injection when the karyokinetic processes have been interrupted and a proportionately large number of the tumor cells are in metaphase. It is not suggested that such approximate averages determine specifically for all cancer cases the time when irradiation should be started, but that an approximate level of activity for a small, non-lethal dose of colchicine should be established.

This phase of the work was done with the collaboration of Dr. G. B. Silver. Dr. Daniel Laszlo was consulted in connection with the medical status of the patients studied. The patients used in this study were suffering from advanced cancer, with surface lesions. The preliminary part of the work consists of establishing a normal metaphase count in several patients to ascertain the relative constancy of the various division phases. In these adjustment studies, no significant fluctuations were noted. Fourteen patients were selected. Of these, half the number yielded suitable material for repeated biopsies and counts. These were: one case of squamous carcinoma of the cervix, one adenocarcinoma of the rectum, one adenocarcinoma of the sigmoid, one carcinoma of the bile duct, two epidermoid carcinomas of the skin, and one adenocarcinoma of the ovary.

An intramuscular injection of 2 mg. of colchicine in 1 ml. of sterile physiological saline was given, distant from the site of the neoplasm. A biopsy was made shortly before the injection, at four hours after the injection, at four-hour intervals for the first 24 hours, and at daily intervals for the following two days. The tissues were fixed and stained. A number of sections were mounted on each slide in serial order. The metaphases counted in 30 high power fields (H.P.F.) were averaged and recorded.

The number of metaphases in the pre-colchicine tissue varied from zero to 1.3 per H.P.F. The first ten hours after colchicine showed a slight increase in the mitotic count. After this period, the number of metaphases increased until a maximum was reached, between the 16th and 24th hours. This was followed by a gradual decline in the number of metaphases. After 72 hours, the number of metaphases was still above that of the pre-colchicine count. There was, in general, a uniform response to the colchicine shown by all the patients tested. The maximum occurred most often at 24 hours after the single injection of the drug. The number of cells that responded varied considerably with the patient or the tumor and ranged from 1.7 to 14.3 per H.P.F. at this period. While the karyokinetic activity varied in number, five cases were proportionately similar. In two cases, cancer of the bile duct and cancer of the sigmoid, the number of metaphases was ten times greater than in any of the other patients studied.

In one of the cases (cancer of the bile duct), partially studied and not included in the series reported above, two biopsies, taken a day apart before colchicine was administered, gave an average of 30 H.P.F. counts of 0.78 and 0.98 metaphases. Sixteen hours after the drug was injected, the average number of metaphases increased to 1.92 per H.P.F. Two months later, this patient was tested again. The control count revealed 1.1 metaphases but, at 16 hours after an injection of colchicine, the count rose to 9.0 per H.P.F.

In another case partially studied, the counts before and after two separate injections seemed significant. In this case, the control biopsy gave an average of 6.1 metaphases. After the colchicine and at 16 hours, the average count was 21.4 metaphases. Following a rest of a week, another biopsy revealed the mitotic activity of 5.6 metaphases. A second injection and an examination of the tissue 16 hours later showed an average count of 39. Ludford (1936) claimed that colchicine effect is cumulative, and Brues

(1942) claimed that 50 per cent of a colchicine injection is recovered from the whole mouse 16 hours after administration. This case suggests an accumulation and retention of the drug. The long intervals between the first and second colchicine installations may indicate the development of a higher sensitivity to the drug.

This study indicates that a small dose of colchicine (2 mg.), injected intramuscularly, is sufficient to arrest nuclear division in metaphase. This dosage is sufficient to increase the number of metaphases for a period of 16 to 24 hours. Following this period, the decline is slow and, at 72 hours after the injection, the number is elevated above the normal unaffected metaphase number. Repeated injections of colchicine indicate possible cumulative and retention effects but seem to show an increased sensitivity because of the long period of rest between two succeeding injections.

Biopsies at 48 and 72 hours after colchicine show some hemorrhage, leukocytosis, some large masses of chromosomes, and polyploidy. Fragmentation, fusion, and clumping of chromosomes were not considered an effect due entirely to the action of colchicine, but a concomitant phenomenon inherent to the disease.

The last stage of this project has been initiated. The possibility of the discovery of other drugs, equally potent and less toxic, has directed attention to such discoveries.

Summary and Conclusions

The action of colchicine on the mechanism of cell division and its effect on growth of normal and neoplastic tissues in humans, animals, and plants is reviewed. The extensive colchicine literature that has developed since Dustin and the members of his laboratory began exploring the effects of various chemicals on karyokinesis is so considerable that a complete summary would be impossible in the allotted space.

However, some of the more recent studies of the effects of colchicine on animal cells have been brought together with the studies made on human cancer and the overgrowth and normal tissue of plants. It appears that the disturbances of the cell mechanism, induced by chemical and physical agents, are alike. Colchicine is perhaps a unique agent. It is extremely toxic in high concentration, yet its level of activity is extremely low and its range of activity is comparatively wide. Though none of the colchicine derivatives is as toxic as colchicine, none is as effective as colchicine at the same concentration.

The effect of X rays and radium emanations (Levine 1926) on the mitosis of animal and plant cells has been known since the turn of the century. The application of irradiations in the treatment of neoplastic diseases of man is recognized as standard procedure, based on the impairment brought about by these physical agents on the mechanics of karyokinesis.

Complete successful achievement of this therapeutic measure alone has never been attained with internal neoplasia. The belief that the chromosomes in the mitotic process are more susceptible to X rays or gamma radia-

tion has directed attention to the use of some agent that would expose the largest number of cells to the action of these ionizing agents.

It was necessary to determine at what period after the administration of colchicine the mitotic phases were most numerous. The application of both agents on a simple plant tissue, uncomplicated by circulating tissues, numerous enzyme systems, and serum reaction phenomena, made this plant tissue the choice for this study. The application of the basic procedures and principles to human cancer was hoped for. Lower forms of mammals had been studied under the influence of colchicine and X rays with inconclusive results. The potentialities of colchicine, especially in combination with other chemical or physical agents, have not been exhaustively studied.

Four phases of the colchicine problem have been planned and, in the above report, three parts are submitted:

(1) The effect of a given dose of the drug on the mitotic phenomena of root tips of the common onion, *Allium cepa*. In a systematic study of the mitotic phases of the root tips of a large series of onions subjected to a constant 0.01 per cent aqueous solution of the alkaloid, it was found by the smear and tissue section techniques of examination of root tips at various intervals after treatment that the maximum number of arrested metaphase stages occurred at the twenty-fourth hour. The colchicine used at this concentration induced root tip hypertrophy, which became permanent and formed a gross characteristic of the treated root.

(2) A comparable study was made on advanced cases of human cancer, following an injection of 2 mg. of colchicine. A single intramuscular injection of 2 mg. of the drug in 1 ml. of physiological saline arrested nuclear division in the metaphase stage. The maximum number of metaphases was attained on the basis of sectioned material of seven suitable patients, between the 16th and 24th hours.

(3) The combined influence of a uniform dose of colchicine and a given dose of X rays (900r, 1500r, or 3000r) was studied in the gross for growth responses measured in terms of root length and microscopic changes to determine the cellular response to these agents. The root tips were studied before colchicine, after colchicine, with colchicine and X rays combined, and with X rays alone.

Irradiation with 900r, following exposure to colchicine for more than 48 hours, prevented further growth. Similar results were obtained with 1500r and 48 hours exposure to colchicine. Irradiation with 3000r and colchicine had no greater effect than with irradiation at this dose alone. The cytological changes induced by colchicine and X ray treatment induced intensified chromosome clumping, fragmentation, and extrusion of chromosomes.

Colchicine seems to sensitize cells to X rays at a time when nuclear reconstruction begins.

The potentialities of colchicine in combination with X rays or other physical or chemical agents have not been exhaustively studied.

(4) The study of the combined effects of colchicine and X rays on neoplastic tissue in man have been initiated. Further studies are in progress.

Bibliography

Amoroso, E. C. 1935. Colchicine and tumor growth. Nature **135:** 266.

Back, A. 1946. The combined effect of colchicine and X rays on the growth of *Allium cepa*. Growth **10:** 375.

Beams, H. W. & T. C. Evans. 1940. Some effects of colchicine upon the first cleavage in *Arbacia punctulata*. Biol. **79:** 188.

Berger, C. A. & E. R. Witkus. 1943. A cytological study of C-mitosis in the polysomatic plant *Spinacia oleracea*, with comparative observations on *Allium cepa*. Bull. Torrey Bot. Club **70:** 457.

Blakeslee, A. F. 1937. Dédoublement du nombre des chromosomes chez les plantes par traitement chimique. Comp. Rend. Acad. Sci. Paris **205:** 476.

Blakeslee, A. F. 1939. The present and potential service of chemistry to plant breeding. Amer. J. Bot. **26:** 163.

Blakeslee, A. F. & A. G. Avery. 1937. Methods of inducing doubling of chromosomes in plants by treatment with colchicine. J. Hered. **28:** 393.

Braungart, D. C. & G. E. Orr. 1942. A cytological study of the effect of colchicine on *Drosophila melanogaster*. J. Hered. **33:** 163.

Brown, N. A. 1939. Colchicine in the prevention, inhibition, and death of plant tumors. Phytopath. **29:** 221.

Brown, N. A. 1942. The effect of certain chemicals, some of which produce chromosome doubling, on plant tumors. Phytopath. **32:** 25.

Von Brücke, F. T. & E. F. von Huéber. 1939. Über die erfolgreiche Behandlung einer Krebsmetastase mit Colchicin und Röentgenbestrahlung. Klin. Wochsschr. **18:** 1160.

Brues, A. M. 1942. The fate of colchicine in the body. J. Clinical Invest. **21:** 646.

Brues, A. M. & E. B. Jackson. 1937. Nuclear abnormalities resulting from inhibition of mitosis by colchicine and other substances. Amer. J. Cancer **30:** 504.

Brues, A. M., B. B. Marble, & E. B. Jackson. 1940. Effects of colchicine and radiation on growth of normal tissues and tumors. Amer. J. Cancer **38:** 159.

Bucher, O. 1939. Zur Kenntnis der Mitose. VI. Der Einfluss von Colchicin und Trypaflavin auf den Wachstumsrhythmus und auf die Zeitteilung in Fibrozytenkulturen. Zeitsch. f. Zellforschung und Mikr. Anat. Abt. A. **29:** 283.

D'Amato, F. 1948. The effect of colchicine and ethylene glycol on sticky chromosomes in *Allium cepa*. Hereditas **34:** 83.

Delcourt, R. 1939. Contribution à l'étude des réactions cellulaires provoquées par la colchicine. Le choc caryoclasique chez les amphibiens. Arch. inter. Méd. Expér. **13:** 499.

Delcourt, R. 1939. Recherches sur les réactions et lésions cellulaires provoquées par la colchicine. Le choc caryoclasique chez les amphibiens. Arch. inter. Méd. Expér. **13:** 719.

Dermen, H. 1940. Colchicine, polyploidy, and technique. Bot. Rev. **6:** 599.

Dermen, H. & N. A. Brown. 1940. Cytological basis of killing plant tumors by colchicine. J. Hered. **31:** 197.

Dixon, W. E. & W. Malden. 1908. Colchicine with special reference to its mode of action and its effect on bone-marrow. J. Physiol. **37:** 50.

Dominici, A. 1932. Peau, syphilis, cancer. Thérapeutique Médicale: V: 358. M. Loeper, G. Milian, *et al.* Masson & Cie, Paris.

Du Bilier, B. & S. L. Warren. 1941. The effect of colchicine on the mitotic activity of the Brown-Pearce rabbit epithelioma. Cancer Res. **1:** 966.

Dustin, A. P. 1934. Contribution à l'étude de l'action des poisons caryoclasiques sur les tumeurs animals. II. Action de la colchicine sur le sarcome greffé, type Crocker, de la souris. Bull. Acad. Roy. d. Méd. d. Belgique **14:** 487.

Dustin, A. P. 1937. La colchicine et ses applications a l'analyse de quelques problèmes de cytophysiologie. Mélanges Jean Demoor: 170.

Dustin, A. P. 1938. L'action des arsénicaux et de la colchicine sur la mitose. La stathmocinèse. Compt. Rent. Assoc. d'Anat. **33:** 204.

Dustin, A. P. & C. Grégoire. 1933. Contribution à l'étude de l'action des poisons caryoclasiques sur les tumeurs animals. I. Action du cacodylate de Na et de la Trypaflavine sur le sarcome greffé, type Crocker, de la souris. Bull. Acad. Roy. d. Méd. d. Belgique **13:** 585.

Eigsti, O. J. 1938. A cytological study of colchicine effects in the induction of polyploidy in plants. Proc. Nat. Acad. Sci. **24:** 56.

Eigsti, O. J. 1939. Effects of colchicine upon the nuclear and cytoplasmic phases of cell division in the pollen tube. Rec. Genet. Soc. Amer. Genetics **25:** 116. 1940.

Eigsti, O. J. 1940. The effects of colchicine upon the division of the generative cell in *Polygonatum, Tradescantia,* and *Lilium*. Amer. J. Bot. **27:** 512.

Eigsti, O. J. 1947. Colchicine bibliography and supplement by P. Dustin. Lloydia **10:** 65.

Gaulden, M. E. & J. G. Carlson. 1947. Action of different concentrations of colchicine on spindle formation and chromosome arrangement as revealed in the living cell. Genetics **32:** 87.

Gavaudan, P. 1938. Sur les tissus à constitution mixte diploïde et polyploide développés chez les végétaux par action de la colchicine. Compt. Rend. Soc. Biol. **128:** 717.

Gavaudan, P. & N. Gavaudan. 1938. Mécanisme d'action de la colchicine sur la caryocinèse des végétaux. Compt. Rend. Soc. Biol. **128:** 714.

Gavaudan, P., N. Gavaudan, & N. Pomriaskinsky-Kobozieff. 1937. Sur l'influence de la colchicine sur la caryocinèse dans les méristèmes radiculaires de l'*Allium cepa*. Compt. Rend. Soc. Biol. **125:** 705.

Gavrilov, W. & D. Von Bistram. 1939. Action de faibles solutions de colchicine sur les fibroblastes du poussin et sur l'épithélium de son iris *in vitro*. Bull. de l'Assoc. franc. pour l'étude du cancer **28:** 319.

Guyer, M. F. & P. E. Claus. 1939. Irradiation of cancer following injection of colchicine. Proc. Soc. Exper. Biol. Med. **42:** 565.

Guyer, M. F. & P. E. Claus. 1940. Destructive effects on carcinoma of colchicine followed by distilled water. Proc. Soc. Exper. Biol. Med. **43:** 272.

Hadorn, E. & H. Niggli. 1946. Mutation in *Drosophila* after chemical treatment of gonads *in vitro*. Nature **157:** 162.

Halberstaedter, L. & A. Back. 1943. Influence of colchicine alone and combined with X rays on *Paramecium*. Nature **152:** 275.

Hall, T. S. 1946. Abnormalities of amphibian development following exposure of sperm to colchicine. Proc. Soc. Exper. Biol. Med. **62:** 193.

Havas, L. 1937. Colchicine, 'phytocarcinomata' and plant hormones. Nature **140:** 191.

Havas, L. 1937. L'action de la colchicine sur le développement du "phytocarcinome" de la tomate. Bull. de l'Assoc. franç. pour l'étude du Cancer **26:** 635.

Havas, L. 1937. Effects of colchicin and of *Viscum album* preparations upon germination of seeds and growth of seedlings. Nature **139:** 371.

Hirshfeld, J. W., R. Tennant, & A. W. Oughterson. 1940. The effect of colchicine and X ray on a transplantable mammary carcinoma in mice. Yale J. Biol. & Med. **13:** 51.

Kostoff, D. 1938. Irregularities in the mitosis and polyploidy induced by colchicine and acenaphthene. Compt. Rend. (Doklady) de l'Acad. Sci. USSR. **19:** 197.

Krythe, J. M. & S. J. Wellensiek. 1942. Five years of colchicine research. Bibliog. Genet. **14:** 1.

Levan, A. 1938. The effect of colchicine on root mitoses in *Allium*. Hereditas **24:** 471.

Levan, A. 1939. The effect of colchicine on meiosis in *Allium*. Hereditas **25:** 9.

Levine, M. 1929. Cytological studies on irradiated tissues. I. The influence of radium emanation on the microsporogenesis of the lily. Proc. Inter. Congress Plant Sciences **1:** 271.

Levine, M. 1945. The effect of colchicine and X rays on onion root tips. Cancer Res. **5:** 107.

Levine, M. 1945. Colchicine and X rays in the treatment of plant and animal overgrowths. Bot. Rev. **11:** 145.

Levine, M. 1945. The effect of colchicine and acenaphthene in combination with X rays on plant tissue. Bull. Torrey Bot. Club **72:** 563; 1946. *Ibid*. **73:** 34; **73:** 167.

Levine, M. 1948. The cytology of the typical and the amelanotic melanoma. Special Pub. N. Y. Acad. Sci. **4:** 177.

Levine, M. & S. Gelber. 1943. The metaphase stage in colchicinized onion root-tips. Bull. Torrey Bot. Club. **70:** 175.

Levine, M. & J. Lein. 1941. The effects of various growth substances on the number and the length of roots of *Allium cepa*. Amer. J. Bot. **28:** 163.

Levine, M. & G. B. Silver. 1947. Mitotic response to colchicine in human cancer. Proc. Soc. Exp. Biol. Med. **65:** 54.

Lits, F. 1934. Contribution à l'étude des réactions cellulaires provoquées par la colchicine. Compt. Rend. Soc. Biol. **115:** 1421.

Lits, F. J., A. Kirschbaum, & L. C. Strong. 1938. Action of colchicine on a transplanted malignant lymphoid neoplasm in mice of the C_3H strain. Amer. J. Cancer **34:** 196.

Lits, F. J., A. Kirschbaum, & L. C. Strong. 1938. Action of colchicine on a malignant lymphoid neoplasm in mice of an inbred strain. Proc. Soc. Exper. Biol. Med. **38:** 555.

Ludford, R. J. 1936. The action of toxic substances upon the division of normal and malignant cells *in vitro* and *in vivo*. Arch. exp. Zellforsch. **18:** 411.
Ludford, R. J. 1945. Colchicine in the experimental chemotherapy of cancer. J. Nat. Cancer Instit. **6:** 89.
Nebel, B. R. 1937. Mechanism of polyploidy through colchicine. Nature **140:** 1101.
Östergren, G. 1943. Elastic chromosome repulsions. Hereditas. **29:** 444.
Oughterson, A. W., R. Tennant, Jr., & J. W. Hirshfeld. 1937. Effect of colchicine on human tumors. Proc. Soc. Exp. Biol. Med. **36:** 661.
Paletta, F. X. & E. V. Cowdry. 1942. Influence of colchicine during methylcholanthrene epidermal carcinogenesis in mice. Am. J. Path. **18:** 291.
Peters, J. J. 1946. A cytological study of mitosis in the cornea of *Triturus viridescens* during recovery after colchicine treatment. J. Exp. Zool. **103:** 33.
Peyron, A., B. Lafay, & N. Kobozieff. 1936. Sur la régression de la tumeur de Shope du lapin sous l'action de la colchicine. Bull. de l'Ass. franç. pour l'étude du cancer **25:** 874.
Peyron, A., B. Lafay, & G. Poumeau-Delille. 1937. Sur la régression du papillo-épithéliome du lapin (tumeur de Shope) sous l'action de la colchicine. Compt. Rend. Acad. Sci. Paris **205:** 378.
Rosendahl, G. 1941. Attempts to induce polyploidy in ferns by treatment with colchicine and observations on polyploid fern prothallia. Planta **31:** 597.
Samartino, G. T. & R. Rugh. 1946. Effects of colchicine on the frog in relation to ovulation and early development. Proc. Soc. Exper. Biol. Med. **63:** 424.
Seed, L., D. P. Slaughter, & L. R. Limarzi. 1940. Effect of colchicine on human carcinoma. Surgery **7:** 696.
Shimamura, T. 1939. Cytological studies of polyploidy induced by colchicine. Cytologia (Tokyo) **9:** 486.
Solacolu, T., M. Constantinesco, & D. Constantinesco. 1939. Action de la colchicine sur les tumeurs végétales provoquées par le *Bacillus tumefaciens*. Compt. Rend. Soc. Biol. **130:** 1148.
Tennant, R. & A. Liebow. 1940. Actions of colchicine and ethylcarbylamine on tissue cultures. Yale J. Biol. & Med. **13:** 39.
Wada, B. 1940. Lebendbeobachtungen über die Einwerkung des Colchicins auf die Mitose, insbesonders über die Frage der Spindelfigur. Cytologia **11:** 93.
Wilbur, K. M. 1940. Effects of colchicine upon viscosity of the *Arbacia* egg. Proc. Soc. Exp. Biol. Med. **45:** 696.
Wolcott, G. B. 1941. Effect of colchicine on a hepatic. J. Hered. **32**: 67.

Discussion of the Paper

Doctor Austin M. Brues (*Argonne National Laboratory, Chicago, Illinois*): I should like to mention a few unpublished experiments on the mode of action of colchicine which were performed several years ago with the assistance of Miss E. B. Jackson. A colorimetric method described by E. Boyland (Biochem. J. 1938; **32:** 1204), utilizing the green color reaction of colchiceine with ferric iron, was found adaptable to its determination in tissues and body fluids after extraction with chloroform. The identification of colchicine as the precursor of colchiceine (by acid hydrolysis) was confirmed by injection of crude extracts into rats after partial hepatectomy and observation of the highly characteristic mitotic changes which occur in this organ. Qualitative microdetermination was also made by the effect on tissue cultures of chick mesenchyme, which is characteristic at 0.1 gamma per ml.

Following intravenous injection, colchicine rapidly disappears from the blood stream. Analyses of plasma at intervals after injection show a volume of dilution of 23 cc. in the 150-gram rat 30 seconds after injection, and of 76 cc. at five minutes. Thus, colchicine reaches beyond the extracellular water in a matter of a minute or so. The volume of dilution exceeds that of

the body water within half an hour. In whole blood, colchicine rapidly distrubutes itself between plasma and cells in the ratio of 5:3.

Analysis of the whole animal plus excreta at six hours shows 80–100 per cent recovery. The extracted colchicine at this time shows the same pattern of toxicity as the original substance, which indicates that the delayed lethality is not due to its conversion to a more toxic compound, as has been suggested.

Excretion occurs through the urine (10–20 per cent) during the period of highest blood level. The urinary loss is greater when colchicine is given intravenously than when it is given subcutaneously. Colchicine is also excreted into the intestine, both by way of the bile (shown by biliary fistula) and, to a lesser extent, directly (into isolated loops of small intestine). Reabsorption from the bowel probably occurs and results in a state, lasting several hours, during which one-half to two-thirds of the injected dose appears within the intestinal tract. Colchicine appears in the bile almost immediately after injection. The high intestinal concentrations may account for the especially marked cytological effects on the intestinal epithelium. Sarcoma 180 failed to show accumulation of colchicine above the average body concentration, excluding intestine.

The delayed death of animals after colchicine injection is of interest. One can administer, intravenously, a dose 40 to 100 times that which causes death within 24 hours, without shortening life to less than six to eight hours. In the mouse, survival is actually prolonged up to 30 hours when the dose is increased in the supralethal range. This appears to be connected with reduced body temperature, as mice will die soon after being warmed up when they are in this state as a result of supralethal doses. These facts suggest that the lethal action of colchicine is associated with a metabolic factor, such as disappearance or accumulation of a critical metabolite. When the LD_{50} is given in two divided doses, its effectiveness is increased. The optimal interval is about 24 hours. The effect of two doses is at least partly cumulative if the interval is as long as 72 hours.

The accumulation of arrested mitoses was found to be determined very critically by both dose and time. In regenerating rat liver, the maximum per cent of mitotic cells was attained between eight and ten hours after injection, with a dose of one microgram per gram body weight. The mitotic index fell off sharply above and below this dose, and the maximum was not always attained with a given dose. It is clear, at least in the case of certain organs, that the "colchicine technique" for estimating mitotic frequency must be considered as only of qualitative value unless very well controlled. Where doses above the optimal are given, the mitotic spindles disappear altogether within an hour and do not return before death. With smaller doses, they disappear and reappear gradually. Supralethal doses usually do not result in a metaphase count exceeding twice normal (in sarcoma 180), suggesting that the onset of prophase is inhibited.

We have been able to confirm the finding (E. Boyland and M. Boyland, 1937, Biochem. J. **31:** 454–460) that in animals bearing experimental sarcoma, the size of the tumor determines the lethal dose. The lethal dose is

also decreased in very young animals (particularly embryos) and in animals undergoing liver regeneration. Considering the time relation between maximum mitotic effect and death, it may be suggested that the lethal effect is related to a metabolic action peculiar to mitotic or growing cells.

Colchicine has been shown to alter tissue oxygen consumption, but only at concentrations much higher than those effective in producing mitotic arrest or in gout therapy. Further studies on the effect of colchicine at lower concentration on various enzyme systems are indicated.

Experiments by B. G. Ferris have utilized the observation of E. G. Kelley (1939, J. B. C. **127:** 73–86) that mitotic figures fix certain basic stains (*e.g.*, toluidine blue) at a lower pH than resting nuclei. The extreme shortening of chromosomes resulting from colchicine treatment suggested that the nucleic acid:protein ratio, which probably determines this reaction, might be further raised by colchicine. Examination of mitoses in colchicine-treated regenerating liver and mouse sarcoma 180 showed no difference in the critical pH for basic staining from normal mitoses in untreated material.

THE INHIBITION OF CELL DIVISION BY SUBSTITUTED PHENOLS WITH SPECIAL REFERENCE TO THE METABOLISM OF DIVIDING CELLS

By G. H. A. Clowes

The Lilly Research Laboratories, Indianapolis, Indiana, and the Marine Biological Laboratory, Woods Hole, Massachusetts

Certain nitro- and halophenols, when used at appropriate concentrations, increase the oxygen consumption of both fertilized and unfertilized sea urchin eggs (*Arbacia punctulata*). With rising concentration of the nitro- or halo-phenol, the oxygen consumption reaches a peak which is sometimes as much as three or four times the normal. With further increase in concentration of the reagent, the respiration gradually falls to a level which is finally well below that of the normal control eggs.

During the initial period of rising respiration, no effect is exerted on the rate of cell division of fertilized sea urchin eggs. At the peak of respiration, the rate of cell division is usually reduced to about fifty per cent as compared with the normal controls and, with a slight further increase in concentration of nitro- or halophenol, cell division is completely suppressed. This block to cell division is freely reversible over a wide range of concentrations, however, since the eggs, when returned to sea water after periods ranging from three to six hours, divide in a normal manner at a normal rate and develop into normal swimming forms.[1–5] Similar results have been obtained with yeast under both aerobic and anaerobic conditions. Under aerobic conditions, with rising concentration of dinitro-o-cresol, the oxygen consumption rises to a peak and subsequently falls to a point below normal. Similarly, under anaerobic conditions, the CO_2 and alcohol production rises to a peak corresponding very closely with the peak in the aerobic system and subsequently falls to points substantially below the normal.[6]

Since dinitro-o-cresol has been employed as the respiratory stimulating agent in a wide variety of experiments, it is possible to compare the respiratory curves obtained with both fertilized and unfertilized sea urchin eggs with those obtained with yeast under aerobic and anaerobic conditions and also with the effect exerted by the same reagent on the speed of reduction of cytochrome, which also rises to a peak and then falls In spite of some differences in temperature and pH at which these experiments were conducted, the concentration of dinitro-o-cresol which gave the peak effect was found to be almost the same for the two series of experiments on marine eggs, the two series of experiments on yeast, and the time required for cytochrome reduction.[6]

A study of the mode of action of these nitro- and halophenols indicates that, when they are used at a sufficiently high concentration, they inhibit growth and development[7] and block the action of di-amino acid oxidase[8] and zwischenferment and cytochrome reductase.[9] Most significant is the observation that, with rising concentration of dinitrophenol, the phosphate uptake by yeast cells is first diminished and then blocked and, ultimately,

a loss of phosphate may occur.[10] It has also been shown that dinitrophenol inhibits nitrogen assimilation[11] and certain other synthetic reactions.[12]

It should be borne in mind that a complete but reversible block to division of sea urchin and other marine eggs and of yeast and other organisms can be effected by various nitrophenols at concentrations at which the respiration is still substantially above the normal. Similarly, the block to the intake of phosphate and nitrogen and the block to various syntheses by yeast and other organisms are effected at concentrations of nitrophenols at which the oxygen consumption is still well above normal. The nitro- and halophenols differ in this respect from anesthetics like ether and chloroform and hypnotics like the barbiturates, which, even though they exhibit a preliminary stimulation of respiration, do not appear to block cell division until the respiration curve is carried below that of untreated, comparable, normal eggs.

Attention may be drawn here to the fact that dinitrophenol and dinitrocresol, which block cell division at a respiratory level well above normal, do not appear to induce complete anesthesia (as indicated by a block to ciliary motion of *Arenicola* larvae and *Arbacia* sperm and a block to the fertilization of treated *Arbacia* eggs by treated sperm) until the concentrations are raised to a level one hundred to two hundred times that required to block cell division and that, at these levels, at which complete anesthesia is induced, the respiration has actually been reduced to a point at or slightly below that of normal, untreated, control eggs.[4, 5, 13]

The block to the intake of phosphorus and to various synthetic processes dependent on phosphorylation appeared to indicate that the nitrophenols, when used at appropriate concentrations, blocked phosphorylation. Recently, Loomis and Lipmann,[14] using a cell-free kidney granule preparation of the type previously employed by Green, Loomis, and Auerbach,[15] have demonstrated that dinitrophenol, when used at appropriate concentrations, can stimulate the oxygen consumption of this system and at the same time inhibit the aerobic phosphorylation. They found that phosphate also, when employed at suitable concentrations, could exert a stimulating effect on the oxygen consumption of this system similar to that exerted by nitrophenol and that the effect of nitrophenol and phosphate used together was only slightly in excess of that of either nitrophenol or phosphate used alone. This block to phosphorylation was found to be reversible.

Lipmann and Loomis, in a verbal communication to the writer, have authorized the statement that the concentrations of individual nitrophenols at which they obtain peaks of respiration in their phosphorylation system tend to correspond with the peaks of respiration previously obtained with fertilized sea urchin eggs.[4, 5] Furthermore, they find approximately fifty per cent suppression of phosphorylation at the peak of respiration, corresponding with the approximately fifty per cent suppression of cell division found at the peak of respiration of fertilized sea urchin eggs. With a slight further increase in the concentration of the nitrophenol, a complete but reversible suppression of phosphorylation is found, corresponding with the reversible suppression of cell division obtained with the fertilized sea urchin eggs.

The purpose of this paper is to review the effects exerted by a series of nitro- and halophenols and other related compounds on fertilized and unfertilized sea urchin and other marine eggs and yeast, in experiments carried out during the period from 1934 to 1940, in the light of the more recent experiments on living cells and cell-free systems referred to above, in which a stimulation of respiration has been found to be associated with a reversible suppression of phosphorylation and various synthetic processes.

In the first place, it should be noted that some nitro- and halophenols exert no effect on either the respiration or cell division of fertilized sea urchin eggs. From the information available to date, it may be stated that if a nitro- or halophenol exerts an effect of some type on respiration, this is invariably associated with a reversible block to cell division over a comparatively wide range and, *vice versa*, that a reversible block to cell division over a considerable range of concentrations is invariably associated with some effect on respiration. The respiration effect, as previously stated, most commonly takes the form of an initial stimulus at relatively low concentrations of the reagent. This is followed by increasing stimulus with increasing concentrations until a peak of respiration is reached, after which a fall in respiration occurs, until a point is reached substantially below that of the normal controls, apparently without any permanent injury of the eggs. Meanwhile, the block to cell division is completely reversible, often over a range of concentration of 1,000 to 1, from the peak of respiration. This may be three or four times the normal, to the point at which the cell is permanently injured, which may be less than half the normal respiratory level.

With certain nitrophenols, however, the respiration, instead of increasing with rising concentration of the reagent, may start to fall and continue to fall without exhibiting a stimulation of respiration at any stage. This fall in the respiration curve is also associated with a reversible block to cell division over a very wide range of concentrations, from the point at which an effect on respiration is first noted until concentrations are reached at which the egg is permanently injured. If a nitrophenol exerting a stimulating effect on respiration is admixed in suitable proportions, with one exerting a depressing effect on respiration, the stimulating effect may be antagonized by the depressing effect and the respiration left at a normal level. The reversible block to cell division in such a case is found to be additive.[16]

The accompanying series of charts (FIGURES 1 to 7*), giving the ratios for respiration and cell division of eggs treated with various nitro- and halophenols as compared with control eggs, have been reproduced from papers published by Krahl and Clowes.[4, 5] For a detailed description, reference must be made to the original papers. The procedure employed was briefly as follows.

Fertilization was performed before the egg suspension was transferred to the Warburg flask. An egg suspension of two per cent by volume in sea water was used and was found to contain approximately 90,000 to 100,000 eggs per cc. Repeated comparisons were made between egg volumes, de-

* FIGURES 1 to 7 have been reproduced from the Journal of General Physiology, Vol. 20, through the courtesy of the editors of the journal.

termined by the hematocrit and hemocytometer methods, and it was shown that the former method gave a constant average result about eight per cent higher than the latter. Since the magnitude of this difference was known and reproducible, the hematocrit method was employed in order to effect a saving of time.

The data on division were obtained and expressed essentially by the method described by Smith and Clowes.[17] The unit is based on the average

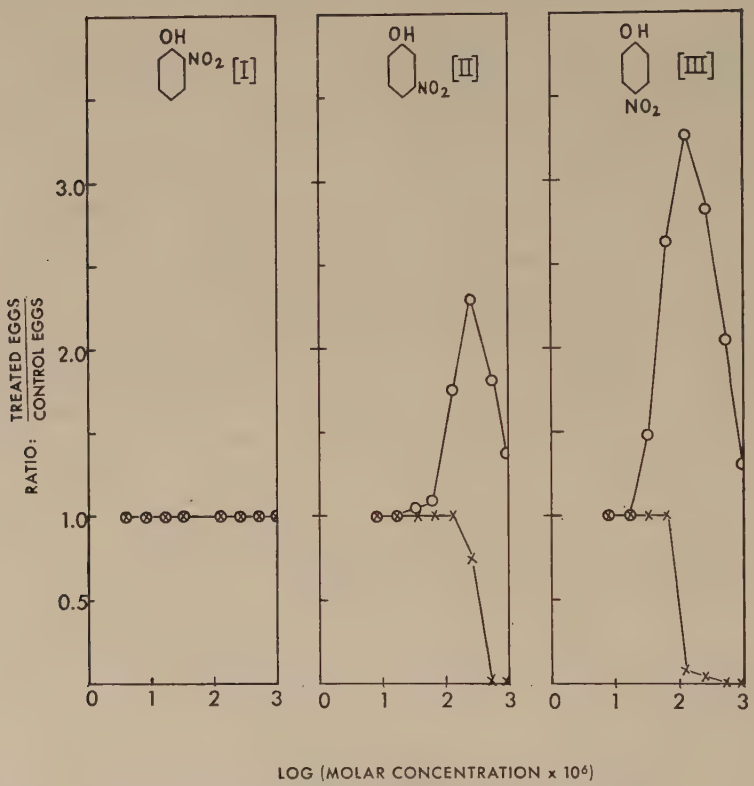


FIGURE 1. Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of o-nitrophenol (I), m-nitrophenol (II), and p-nitrophenol (III) at 20° C. Reagents added 15 minutes after fertilization. ○—○ = O_2 consumed in treated eggs/O_2 consumed in control eggs; ×—× = cell division in treated eggs/cell division in control eggs.

number of divisions in 100 or more eggs. An egg which has divided to two cells has performed one division, an egg divided to four cells two divisions, an egg divided to eight cells three divisions, *etc.* In all curves, the ratio of the respiration of treated eggs over that of control eggs or the ratio of cell divisions of treated eggs over those of control eggs is plotted against a logarithmic curve of the concentrations of the nitro- or halophenols employed. In the cell division curve, a distinction is made between a reversible and an irreversible division block, a solid line being used to denote the first, and a dotted line being used to denote the second type of action.

It will be noted from FIGURE 1 that orthonitrophenol exerts no effect on either respiration or cell division. Metanitrophenol exerts a moderate effect and p-nitrophenol a marked effect on respiration and both m- and p-nitrophenol give a reversible block to cell division, the fifty per cent level corresponding approximately with the peak of respiration.

It will be noted from FIGURE 2 that 2,4-dinitrophenol and 2,6-dinitrophenol exert a somewhat greater effect on both respiration and cell division than do the mononitrophenols, that trinitrophenol (picric acid) exerts no

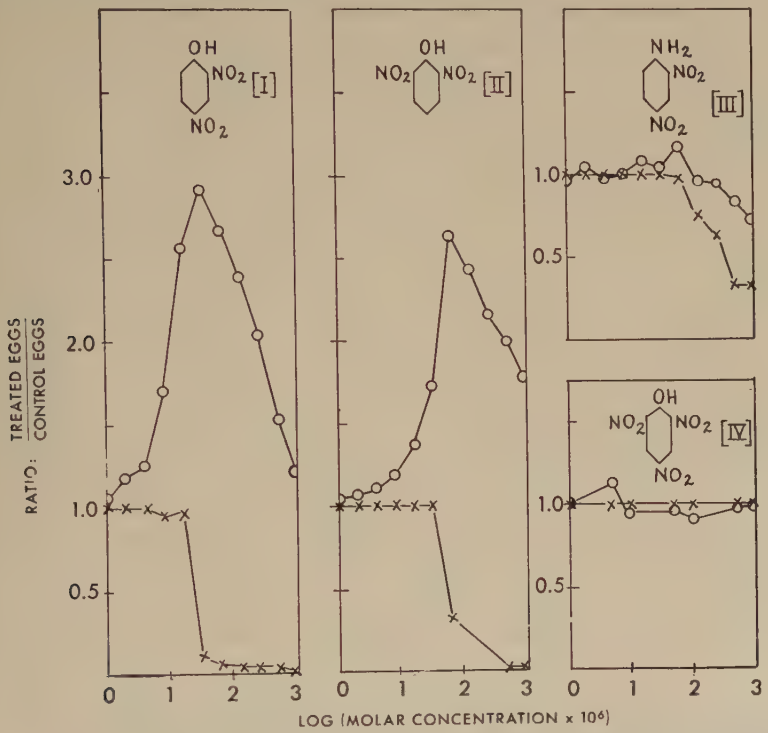


FIGURE 2. Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 2,4-dinitrophenol (I), 2,6-dinitrophenol (II), 2,4-dinitroaniline (III), and 2,4,6-trinitrophenol (IV) at 20° C. Reagents added 15 minutes after fertilization. ○—○ = O_2 consumed in treated eggs/O_2 consumed in control eggs; ×—× = cell division in treated eggs/cell division in control eggs

effect on respiration or cell division, and that 2,4-dinitroaniline gives only a very slight effect on either respiration or cell division.

It will be noted from FIGURE 3 that under comparable conditions 4,6-dinitro-o-cresol exerts a greater effect on both respiration and cell division than does the corresponding 2,4-dinitrophenol and that the peak of respiration falls markedly with rising temperatures, while the block to cell division is not greatly affected.

It will be noted from FIGURE 4 that, while 4,6-dinitrocarvacrol markedly stimulates respiration and suppresses division in a concentration range corresponding very closely with that of the corresponding dinitrocresol, its

isomer, dinitrothymol, exerts no stimulating effect whatever on respiration but instead, causes a depression of respiration and a reversible block to cell division at a concentration range substantially lower than that at which 4,6-dinitrocarvacrol shows a peak of respiration and a block to cell division. It also shows that compounds in which NO or NH_2 are substituted for NO_2 exert very little effect on respiration and fail to give a reversible suppression of cell division.

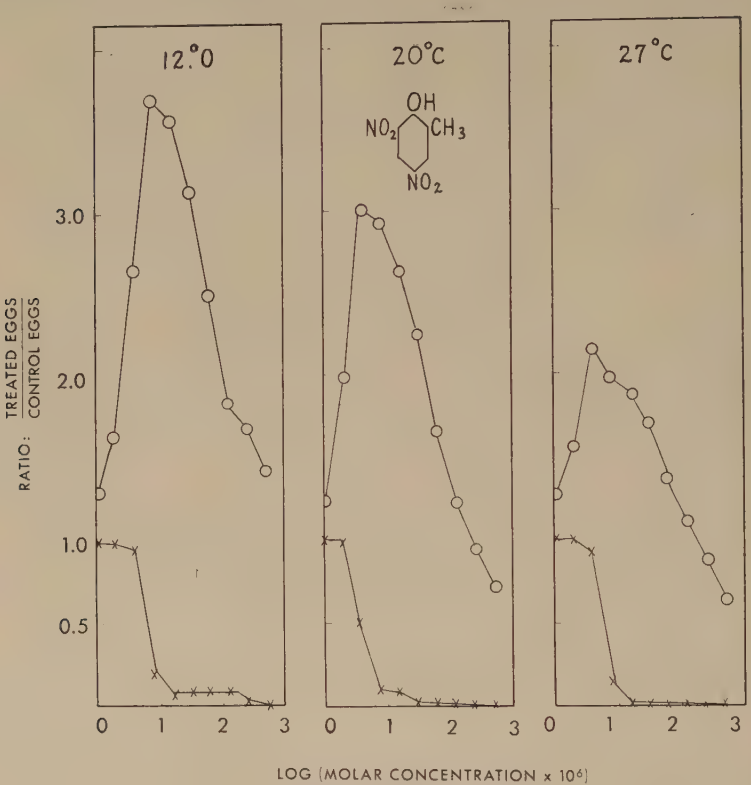


FIGURE 3. Stimulation of oxygen consumption and block to cell division in fertilized *Arbacia* eggs produced by various concentrations of 4,6-dinitro-o-cresol at 12° C., 20° C., and 27° C. Reagent added 15 minutes after fertilization. O—O = O_2 consumed in treated eggs/O_2 consumed in control eggs; X—X = cel division in treated eggs/cell division in control eggs.

A peak of respiration and a reversible suppression of cell division were given by 2,4-dinitro-α-naphthol and by 2,4-dinitro-*ar*-tetrahydro-α-naphthol at dilutions approximately those at which 2,4-dinitrothymol gave a reversible block to cell division. Only a slight effect was given by p-nitroanisole, which may have been attributable to a partial hydrolysis of the methoxy group. No effect was exerted by p-nitrobenzyl alcohol on either respiration or cell division.

Turning now to the halogenated phenols, no monohalophenol was found to exert any effect whatever on either respiration or cell division.

It will be noted from FIGURE 5 that the 2,4-dichloro- and dibromophenols exerted a characteristic effect on respiration and cell division corresponding fairly closely with that exerted by p-nitrophenol, while the 2,6-dibromophenol exerted little or no effect on either respiration or cell division.

It will be noted from FIGURE 6 that the 2,4,6-trichloro-, tribromo-, and triiodophenols all exert an effect on both respiration and cell division, in

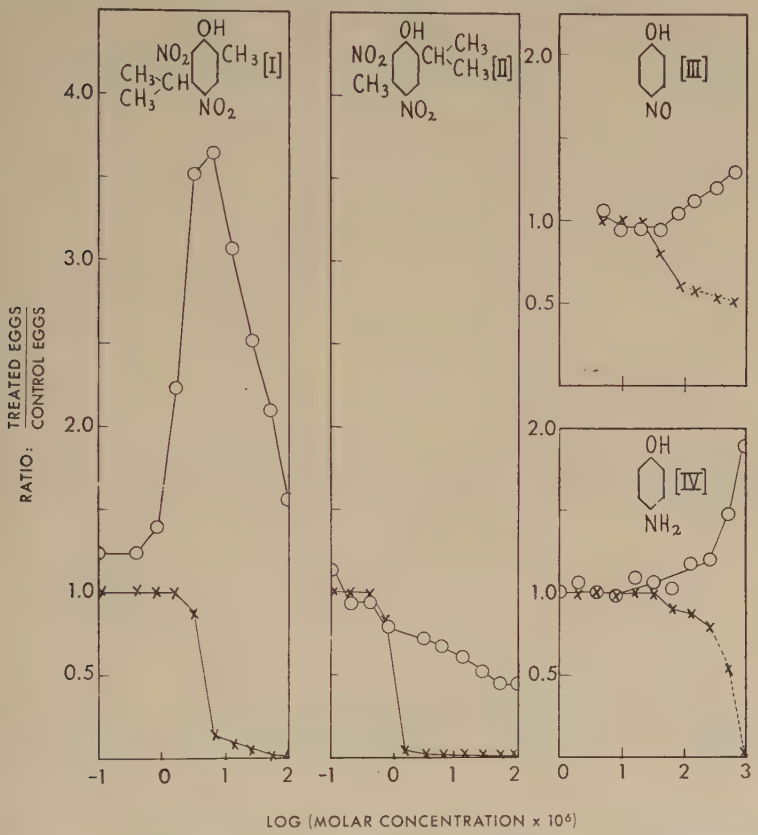


FIGURE 4. Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 4,6-dinitro-carvacrol (I), 2,4-dinitrothymol (II), p-nitrosophenol (III), and p-aminophenol (IV) at 20° C. Reagents added 15 minutes after fertilization. ○—○ = O_2 consumed in treated eggs/O_2 consumed in control eggs; ×—× = cell division in treated eggs/cell division in control eggs.

contradistinction to the corresponding 2,4,6-trinitrophenol, which exerts no effect on respiration or cell division.

As regards the concentration at which the peak of respiration and block to cell division occur, the tribromo- exerts an effect at a greater dilution than the trichloro-, and the triiodo- exerts an effect at a greater dilution than the tribromo-. All trihalophenols thus far investigated exert an effect on both respiration and cell division.

It is interesting to note from FIGURE 7 that a series of mixed nitro- and

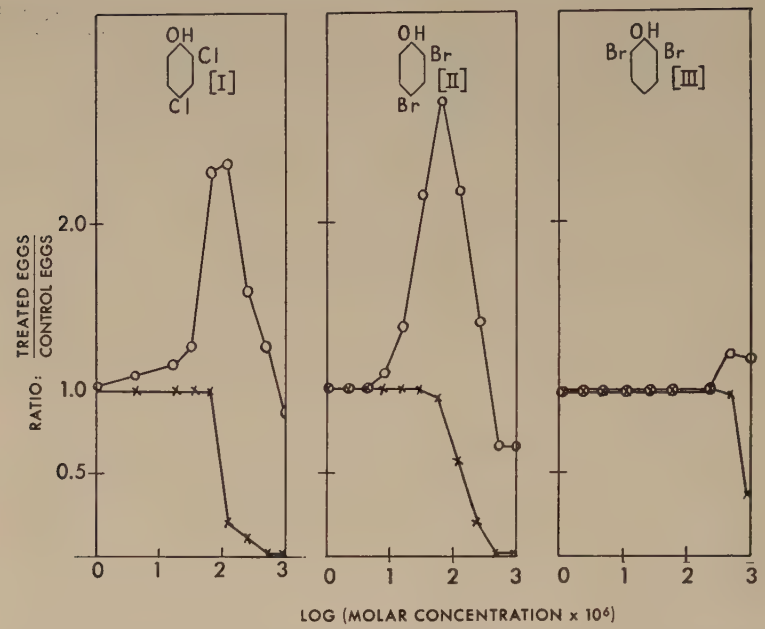


FIGURE 5. Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 2,4-dichlorophenol (I), 2,4-dibromophenol (II), and 2,6-dibromophenol (III) at 20° C. Reagent added 15 minutes after fertilization. ○—○ = O_2 consumed in treated eggs/O_2 consumed in control eggs; ×—× = cell division in treated eggs/cell division in control eggs.

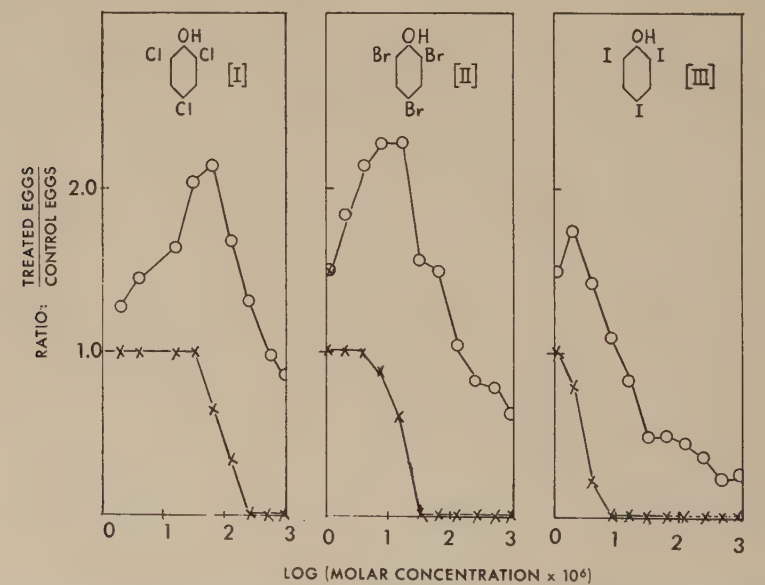


FIGURE 6. Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 2,4,6-trichlorophenol (I), 2,4,6-tribromophenol (II), 2,4,6-triiodophenol (III) at 20° C. Dotted division lines denote irreversible injury. Reagent added 15 minutes after fertilization. ○—○ = O_2 consumed in treated eggs/O_2 consumed in control eggs; ×—× = cell division in treated eggs/cell division in control eggs.

halophenols exert a characteristic stimulating effect on respiration and a reversible suppression of cell division. In this regard, the 2,6-dinitro-4-chlorophenol appears to act in a very similar manner to trichlorophenol and in marked contrast to the inactive 2,4,6-trinitrophenol.

In addition to the above, tests were conducted with o-aminophenol, o-nitroanisole, p-nitrophenetole, p-nitrobenzonitrile, p-nitroacetonitrile, p-nitrophenylacetic acid, p-nitrobenzamide, p-nitrophenylisocyanate, o-nitroaniline, p-nitroaniline, p-nitrodimethylaniline, p-nitrodiethylaniline, p-nitrosodimethylaniline, p-nitrosodiethylaniline, o-dinitrobenzene, p-dinitrobenzene, o-nitrochlorobenzene, m-nitrochlorobenzene, and p-nitrochloroben-

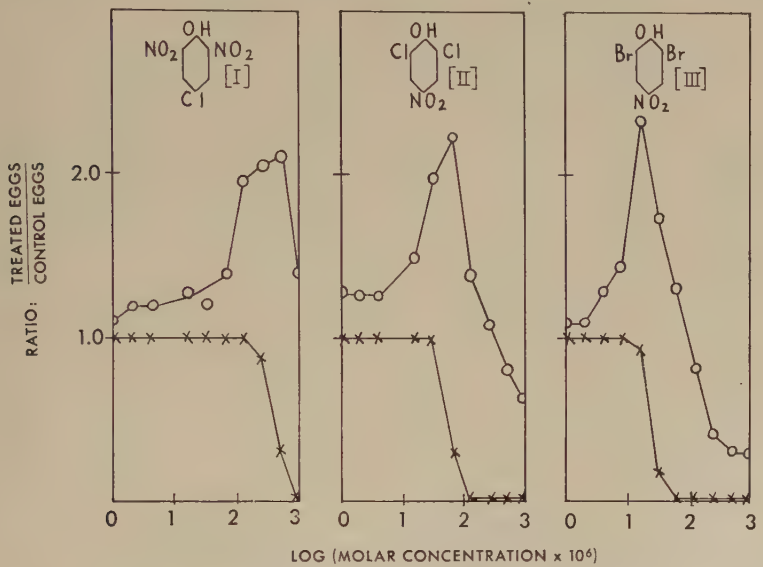


FIGURE 7. Stimulation of oxygen consumption and block to cell division of fertilized *Arbacia* eggs produced by various concentrations of 2,6-dinitro-4-chlorophenol (I), 2,6-dichloro-4-nitrophenol (II), 2,6-dibromo-4-nitrophenol (III) at 20° C. Reagents added 25 minutes after fertilization. O—O = O_2 consumed by treated eggs/O_2 consumed by control eggs; X—X = cell divison in treated eggs/cell division in control eggs.

zene. These compounds proved to be ineffective in producing either a marked respiratory stimulation or a reversible division block over any wide range of concentrations. The dinitrobenzenes, which lack the phenolic hydroxy group, exert no effect on cell division until very high, and usually lethal, concentrations are reached.

A series of reversible oxidation-reduction indicators, o-cresol indophenol, methylene blue, neutral red, dimethyl-p-phenylene diamine, tetramethyl-p-phenylene diamine, and pyocyanine, exerted some stimulating effect on respiration and a block to cell division, but the block to cell division was in no sense reversible. It must be concluded that fertilized sea urchin eggs subjected to optimum respiratory-stimulating concentrations of these dyes are, for all intents and purposes, dead cells.

From experiments carried on with upwards of sixty compounds, the con-

clusion has been reached that a truly reversible block to cell division over a wide range of concentrations, on a respiratory curve that is falling but is still well above that of the normal control, is obtainable only with compounds containing the phenolic hydroxy group associated with nitro- and/or halo-groups in appropriate numbers and locations. It is particularly interesting to note that o-nitrophenol exerts no effect on respiration or cell division, while the m- and p- compounds exert a characteristic effect on both, that all the dinitrophenols exert an effect on both respiration and cell division somewhat greater than that of the mononitrophenols, but trinitrophenol (picric acid) exerts no effect on either respiration or cell division. With the halogenated phenols, the situation is somewhat different. All the mono-, chloro-, bromo-, and iodophenols thus far tested have been entirely negative. The dihalogenated phenols, with the exception of the 2,6- compounds, exert an effect on both respiration and cell division, as do all the trihalogenated phenols and the combinations of mono-halogen-dinitro- or mono-nitro-dihalogenated phenols. The effect of alkyl groups introduced into the molecule is extremely interesting. The introduction of the methyl group in the ortho position makes p-nitrocresol more active and more effective on both respiration and cell division block than is the p-nitrophenol, and the dinitrocresol is similarly more active than the dinitrophenol. Finally, the introduction of an isopropyl group in the ortho position in dinitrophenol, with a methyl group in the 5 position, gives an effect on reversible cell division block somewhat greater than that of the corresponding dinitrocresol.

While the acidity of the phenolic group is undoubtedly significant, it certainly is not the sole factor in determining the extent of respiration stimulus and reversible block to cell division exerted by given nitro- and halophenols. A tabulation[18] of a series of substituted phenols, based on the concentration required to produce fifty per cent reduction of the rate of cell division of fertilized *Arbacia* eggs, shows no consistent relation to pK values. It is found that those compounds which require the smallest concentrations of anion are chiefly those with pK values between 3.5 and 4.5. Since 2,4,5-trichlorophenol, with a pK of 6.9, falls near the top of the table, however, and 2,6-dinitro-4-chlorophenol, with a pK of 3.5, falls near the bottom, it is evident that no final conclusions can be based on pK values alone. It should be noted that compounds which require the smallest anion concentrations tend to be rather large molecules which have, in the undissociated form, a lower water solubility and a higher lipoid water distribution coefficient than the compounds falling lower in the scale. The failure of 2,4,6-trinitrophenol to exert a stimulating effect on respiration or a reversible block to cell division may be explained on the basis of its high acidity, as indicated by its pK value of 0.7, and the fact that the trihalophenols and dihalomononitro- and dinitromonohalophenols all exert an effect on both respiration and cell division may be attributed to the fact that, in contradistinction to the trinitrophenol, their pK values fall within the range in which definite respiratory and cell division blocking effects may be anticipated. The fact that o-nitrophenol, which is absolutely inactive, and p-nitrophenol, which exerts a pronounced effect on both res-

piration and cell division, both have a pK value of approximately 7.2 may possibly be explained on the basis of the known chemical effect of certain groups placed in the ortho- position to the hydroxyl in the benzene ring. A similar explanation may be afforded for the inactivity of the 2,6-dihalophenols.

In considering the effect of the acidity of the phenolic group, it should be noted that only those substituted phenols with pK values ranging from 3.5 to 4.5 or 5.0 appear to be capable of stimulating the metabolism of intact mammals. This may be attributable to the relatively large mass of tissue in relation to the external aqueous phase and the consequent difficulty experienced in providing a sufficiently high ionic concentration of the substituted phenol in the interior aqueous protoplasmic phase within ranges that are nonlethal, on account of the relatively large proportion of the highly lipo-soluble substituted phenol molecules taken up by the lipoid and other nonaqueous components of the cell.

With general anesthetics, the amounts required to induce a given anesthetic effect, for example, the suppression of motility of larvae or block to fertilization, tend to increase with an increase in the number of larvae employed in a given volume of suspending medium. In the case of the nitrophenols, the amounts required to anesthetize larvae and block fertilization rise with an increase in the number of eggs, but to a lesser extent than with general anesthetics. The concentration required to block cell division appears to be independent of the number of eggs, but rising concentrations of CO_2 with greatly increased numbers of eggs introduce a complicating factor.

The concentrations of certain nitrophenols required to suppress motility of *Arenicola* or *Arbacia* larvae are far greater than the concentrations required to block cell division and appear to bear some relation to the acidity of the compounds in question, the differential between the concentrations required to produce anesthesia and those required to block cell division tending to be greater in the 3.5 to 4.5 pK ranges and to diminish with rising pK values.[13]

The effect of alkyl groups, in profoundly modifying the effect on both respiration and reversible cell division block, is reminiscent of the effect of introducing alkyl groups into polycyclic hydrocarbons, and, by the location and distribution of such groups, increasing or decreasing the carcinogenic effect of the compounds in question and also modifying the facility with which these compounds are held in a monomolecular sterol film at an air-water interface.[19]

The above considerations, and, particularly, the complete reversibility of the cell division block, often over a range of concentrations of 1,000 to 1, suggest the likelihood that these nitro- and halophenols exert their blocking effect by blocking certain active enzymatic or complex molecular surfaces in the protoplasmic structure. The complete reversibility of the cell division blocking effect over a very wide range of concentrations exhibited by certain nitro- and halophenols appears to rule out oxidation-reduction or acid-basic types of reaction which, at the higher concentration employed,

might be expected to modify or denature the proteins and suggests the likelihood that these nitro- and halophenols effect a molecular surface block by hydrogen bonding and van der Waals residual valence effects of the type studied by Pauling in connection with the development of immune and other specific phenomena. The observation that, while the concentration at which dinitrocresol gives a peak respiratory effect and a fifty per cent block to division is apparently unchanged with temperature, the extent of respiratory stimulus, as measured by the ratio of respiration at the peak to that of the untreated controls, drops from 3.7 at 12° to 3.0 at 20° and 2.1 at 27° may lend some support to the above-suggested viewpoint, since adsorption tends to decrease with rising temperature.[4]

The possibility that nitro- and halophenol anions exert a stimulating effect on respiration, in contradistinction to the unionized molecule, which depresses respiration and blocks cell division, thus accounting for the peak effect in respiration and the relation of this possible mechanism to the falling peaks of respiration with rising temperature, will be discussed later.

In attempting to find an explanation for the fact that, with nitro- and halophenols, the rate of cell division is reduced to fifty per cent of the normal at the peak point of respiration, even though the oxygen consumption may be three or four times the normal, it is interesting to note that, in a series of experiments in which the oxygen ratio to nitrogen in the atmosphere was lowered without addition of nitro- or halophenol to the point at which the oxygen consumption by the eggs was reduced to half the normal, the rate of cell division was found to be lowered fifty per cent. When the oxygen consumption was cut to 32 to 35 per cent of the normal, cell division was entirely suppressed.[20] Similar results were obtained when respiration was partially blocked by means of carbon monoxide, the rate of division being cut to fifty per cent at the fifty per cent oxygen consumption level and completely suppressed at the thirty-five per cent oxygen consumption level.

When KCN is employed to suppress oxygen consumption, a similar retardation of division is observed, with complete block to division when the oxygen consumption is reduced to thirty-five per cent. In this series of experiments, either with reduced oxygen tension or block to oxygen consumption by CO or KCN, it should be noted that, when the oxygen supply is diminished so as to lower gradually the oxygen consumption, the addition of nitrophenol exerts less and less stimulating effect on oxygen consumption, until, finally, no stimulation whatever is observed.[21] In these cases, the block to cell division, as a result of oxygen lack on the one hand, and the action of nitrophenol on the other, is found to be additive. The portion of the nitrophenol respiration curve preceding the point of maximum stimulation is far more sensitive to the action of cyanide than the subsequent falling portion of the curve.

The above data, considered in conjunction with the fact that certain nitrophenols, which exert no stimulating effect on respiration but only a depressing effect, reversibly block cell division over a very wide range of concentrations, would appear to indicate that the rising portion of the res-

piratory curve has nothing to do with the block to cell division but that this latter effect is associated exclusively with the dropping portion of the curve. In fact, the observation that the rising portion of the respiration curve is far more sensitive to the action of cyanide than the subsequent falling portion of the curve would appear to indicate that a reversible cell division block is only encountered when the concentration of nitrophenol is high enough to affect that portion of the respiratory mechanism that is least sensitive to cyanide.

It would appear that the increased energy produced as a result of the greatly increased consumption of oxygen and production of carbon dioxide effected by the majority of active nitrophenols at low concentrations is serving no useful purpose and is being in great part dissipated as heat. In fact, since the rate of cell division, and also the rate of phosphorylation, are reduced to a fifty per cent level at the peak of respiration and suspended altogether at a slightly higher level, which, in the absence of nitrophenols, appears to be that at which the normal oxygen consumption is reduced to thirty to thirty-five per cent of the normal, it may reasonably be assumed that, not only is all the surplus energy resulting from the increased oxygen consumption being dissipated, but so also is a portion and, finally, with rising concentration of the nitrophenol, the whole of the energy normally required for phosphorylation, various synthetic processes, and cell division.

A reference has previously been made to the possible role of the nitro- and halophenol ions as respiratory stimulants. An extensive series of experiments was carried out in which the effect of five representative substituted phenols on the respiration and cell division of fertilized sea urchin eggs was determined at constant cytoplasmic pH and constant extracellular pH, at varying extracellular pH with varying cytoplasmic pH, at constant cytoplasmic pH with varying extracellular pH, and at constant extracellular pH with varying cytoplasmic pH.[22] These varying conditions may be established by the exterior use of either a glycyl glycine buffer, which does not penetrate, or a CO_2 bicarbonate buffer, the CO_2 of which can penetrate the egg, or by changing the partial pressure of CO_2 to which the eggs are exposed, or by introducing ammonium salts into the system.[23] Interiorly contained CO_2 tends to lower, and NH_3 to raise, the pH of the interior aqueous phases of cell protoplasm. On the basis of theoretical considerations, for which reference must be made to the original paper,[18] when equilibrium is finally established, the concentration of unionized substituted phenol molecules in the interior aqueous phase of the cell protoplasm is equal to that in the exterior aqueous medium, regardless of ionic shifts caused by variations in the pH of the interior aqueous protoplasm effected by introduction of CO_2 on the one hand or NH_3 on the other or of the concentration of unionized substituted phenol molecules combined with, dissolved in, or adsorbed on lipoidal or lipo-protein components of the cell protoplasm.

When the external pH was held constant and the interior pH shifted, by using a glycyl glycine buffer on the one hand and a carbon dioxide bicarbonate buffer on the other, the peak of respiration and fifty per cent block to cell division were effected at approximately constant concentrations in a

series of five typical nitro- and halophenols, but the stimulation of respiration and the peak of respiration were lowered to a very great extent in the case of dinitrophenol, having a pK value of 4.1, and dinitrocresol, having a pK value of 4.4. The respiration stimulus and peak of respiration were lowered to a lesser extent in the case of trichlorophenol, having a pK value of 6.9, and little or no change was effected with dichlorophenol, having a pK value of 7.7, and m-nitrophenol, having a pK value of 8.3.

A further example, in which the same series of nitro- and halophenols exerted effects in the same order, is that in which both the exterior and the interior pH values were shifted by subjecting the eggs to the action of different concentrations of the nitro- and halophenols at each of three levels of CO_2 partial pressure. With rising CO_2 concentrations, the extent of respiratory stimulus and the height of respiration peaks were markedly decreased, and the concentration at which the respiration peak and the fifty per cent block to cell division were observed was markedly lower in the case of dinitrophenol and dinitrocresol, having pK values of 4.1 and 4.4, respectively. On the other hand, the decrease in respiration and respiration peak was far less, and the shift in fifty per cent block to cell division was practically negligible in the case of the trichlorophenol, the dichlorophenol, and the m-nitrophenol, having pK values of 6.9, 7.7, and 8.3, respectively.

These and other results in which the interior aqueous pH values were shifted down by means of CO_2 and up by means of ammonia were all explicable only on the assumption that the nitro-and halophenols penetrated the cell only as unionized molecules, that stimulation of respiration was dependent on the concentration of ions in the interior aqueous protoplasmic phase and the suppression of respiration and block to cell division by the concentration of unionized molecules at some point in the interior protoplasmic system. Since the concentration of substituted phenol ions in a nonaqueous phase is negligible, these ions must exert their stimulating effect in or at the surface of interior aqueous protoplasmic phases, presumably by interaction with, or adsorption on, complex molecular surfaces which are wetted by the aqueous phase.

On the other hand, certain nitrophenols, particularly some of those having pK values in the range from 3.5 to 5.0, gave a commencing suppression of respiration and an initial block to cell division at dilutions which, on account of their low pK values, would show molecular concentrations in interior aqueous protoplasmic phases below 10^{-10} M. Since this represents a negligible concentration in the water phase, it is obvious that the point of action of the intact molecule cannot be in the water phase. Furthermore, certain of these nitrophenols which exhibit a maximum capacity to block cell division are virtually insoluble in water and have a relatively very high lipo-water solubility coefficient. This indicates that the unionized molecules, when present in the cell, are probably associated in great measure with lipoids in lipo-protein and other relatively nonaqueous phases of the protoplasmic system. It may be concluded, therefore, that the ions and the unionized molecules react with and are adsorbed either on altogether

different molecular interfaces, or in a different manner on the same interface, and consequently exert entirely different effects.

Similar experiments carried out with a series of barbiturates,[24] on the one hand, and a series of local anesthetics,[25] on the other, indicated that both the barbiturates and the local anesthetics penetrated the cell as unionized molecules. However, the anesthetic effect of the barbiturates in suppressing both cell division and ciliary action appeared to be attributable entirely to the unionized barbituric acid, while the similar anesthetic effect exerted by the local anesthetics appeared to be attributable entirely to the ionized cation.

The series of results obtained with nitro- and halophenols, barbiturates, and local anesthetics by shifting the pH of the interiorly contained protoplasmic aqueous phases, while keeping the pH of the exterior medium constant, were so uniformly consistent as to render it extremely probable that the conclusion that the nitro- and halophenol ion is responsbile for respiratory stimulus and the molecule for suppression of respiration and cell division is correct.

The nitro- and halophenols that block respiration are also capable of suppressing the motility of larvae and sperm and the fertilization process when used at sufficient concentrations, which may be far in excess of those required to block cell division. In a series of experiments carried out in solutions buffered at pH 8.0, the motility of sperm and the ciliary movement of *Arbacia*, *Arenicola*, and other larvae are first suppressed in concentrations of 2,4-dinitrophenol and 4,6-dinitro-o-cresol, 137 and 228 times, respectively, that required to suppress cell division. The fertilization of *Arbacia* eggs by sperm, as evidenced by the development of a fertilization membrane, also takes place in the presence of slightly lower concentrations of these nitrophenols. While subsequent cell division is suppressed, the fact that fertilization has actually taken place may be demonstrated by washing and centrifuging the eggs several times with solutions of sea water containing the concentration of nitrophenol in which fertilization still occurs, until all supplementary sperm have been removed. The eggs, when returned to sea water, develop at a normal rate to normal swimming forms, leaving no doubt that true fertilization was effected by sperm in the presence of concentrations of nitrophenols from at least 100 to 200 times that required to suppress cell division.

Ciliary movement of *Arenicola* larvae, motility of *Arbacia* sperm, and fertilization of treated *Arbacia* eggs by treated sperm are first inhibited by 2,4,5-trichlorophenol at concentrations far below those required for a similar purpose in the case of 2,4-dinitrophenol and 4,6-dinitro-o-cresol. However, the concentration at which cell division of fertilized *Arbacia* eggs is blocked lies between the blocking concentrations of the two above-mentioned nitrophenols. As a result, the ratio between the concentration required to block fertilization and that required to block cell division is in the order of twenty to one instead of two hundred to one. The use of o-nitrophenol exerts no effect on ciliary movement or fertilization, just as it exerts no effect on cell division. 2,4-Dichlorophenol exerts an effect on ciliary move-

ment and the fertilization of treated eggs by treated sperm in concentrations that are far below those required for the dinitrophenols but requires a substantially higher concentration to block cell division of fertilized *Arbacia* eggs, with the result that the ratio between the concentrations required to block fertilization and those required to block cell division become approximately one to one. Again, m-nitrophenol blocks ciliary movement and fertilization at about the same concentration as is required by 2,4-dinitrophenol and 4,6-dinitro-o-cresol, but the concentration required to block cell division of fertilized *Arbacia* eggs is more than fifty times as great as that required by 4,6-dinitro-o-cresol, giving a ratio between the concentrations required to block fertilization and cell division in the order of magnitude of four to one, or less.

In considering the above data, it is important to note that the pK values of 2,4-dinitrophenol and 4,6-dinitro-o-cresol are, respectively, 4.1 and 4.4, that of 2,4,5-trichlorophenol 6.9, and those of 2,4-dichlorophenol and m-nitrophenol, respectively, 7.7 and 8.3. This ratio between the concentrations of nitro- and halophenols required to produce complete anesthesia, as evidenced by suppression of motility of larvae and sperm, fertilization, *etc.*, and those required to suppress cell division, ranging from 100 to 200 to 1 for substances having pK values of 4 to 4.5, down through 20 to 1 for those having pK values between 6 and 7, and finally reaching a ratio approaching unity for substances having pK values at or near 8, is obviously highly significant. It apparently follows a similar differential order to that observed in the case of the previously reported experiments in which pH changes were effected in the interior protoplasm and the exterior medium, in which, also, the differentials were always most readily observed with substances having pK values in the range of 4 to 4.5.

It should be remembered that the suppression of cell division depends on the suppression of the active process of phosphorylation and subsequent syntheses, which may well be far more sensitive than the mechanism concerned with general anesthesia, which may depend on the attainment of a sufficiently high concentration of the unionized nitro- or halophenol molecules in solution in, or in contact with, lipo components of the cell, particularly in the cell membrane. Also, it should be remembered that the anesthesia experiments were carried out in a medium with a pH of 8, while the pH of the cytoplasm in which the phosphorylation mechanism is presumably functioning is probably somewhat under 7. It has been previously noted that the concentration of nitrophenol required to suppress cell division appears to be independent of the number of eggs employed, while the concentration of nitrophenol required to anesthetize larvae and block fertilization appears to rise with the number of eggs, but not as rapidly as in the case of general anesthetics. It has already been suggested that this block to cell division, being reversible over a very wide range of concentrations, frequently as great as 1,000 to 1, may well be attributable to adsorption of a unimolecular layer of the reagent concerned on some enzymatic protoplasmic interface by means of hydrogen bonding and van der Waals effects, which might be expected to be fully reversible, and which would

obviously call for a relatively small number of molecules to exert a given inhibiting effect.

It should also be remembered that, since these most striking inhibitant effects are obtained with nitrophenols having pK values in the range from 3.5 to 5 at concentrations at which the respiration is still maintained at a high level, often three times the normal, the local concentration of carbon dioxide in the vicinity of the enzymatic surface concerned may be abnormally high and may readily induce a greatly increased acid effect in the vicinity of, or on, the protoplasmic molecular surface concerned. This might well result in a greatly increased concentration of nitrophenol molecules in the vicinity and on the surface in question.

Finally, from the experiments of Loomis and Lipmann, it appeared possible that nitrophenol molecules might be substituted for phosphate molecules at least so far as the respiratory phase of phosphorylation is concerned. This suggests the possibility that such nitrophenol molecules, substituting for phosphate in a phosphorylating system while stimulating respiration up to a given concentration, might ultimately block respiration and phosphorylation so far as the synthetic steps leading up to growth and cell division are concerned. In any case, further speculation on this question would appear inadvisable until an attempt has been made to obtain from sea urchin eggs a cell-free, isolated, phosphorylating system of the type employed by Loomis and Lipmann, which, if subjected to varying concentrations of various nitro- and halophenols and phosphate, varying pH and temperature, and varying oxygen and CO_2 tension, should throw substantial light on this and other problems suggested in this review.

The cyclophorase system obtained by Green, Loomis, and Auerbach and described by them as containing nucleo-protein and possibly consisting of a mosaic containing various enzymes intimately interrelated with one another, is obviously a heterogeneous system which is highly sensitive to dehydration or any other physical or chemical steps which may irreversibly modify the interrelation of the various components of the system. The cyclophorase is present in a sol and tends to lose its efficacy when the sol is precipitated as a curd. Its destruction by freezing, drying, or exposure to concentrations of salt greater than 0.1 N, to solvents such as alcohol or acetone in concentrations greater than ten per cent by volume, to acidity below pH 4, or to alkalinity above pH 10 is suggestive of the type of heterogeneous system postulated above, in which a particular, essential interrelation between given proteins, lipoidal components, and electrolytes is easily and irreversibly destroyed by dehydration or any procedure tending in any way to modify the specific and essential interrelation of the component parts. It would appear that a full explanation of some of the observations reported in this review must await further information regarding the nature of the components of these phosphorylating systems and the manner in which they are interrelated.

The suppression of cell division by nitrophenols tends to occur in the prophase. If, under fixed conditions of experiment, uniform samples of fertilized *Arbacia* eggs are removed at five-minute intervals during the

first three divisions and put into a suitable fixative, the number of eggs found to be in the prophase will be about twenty-five per cent. If, however, identically comparable eggs, in the same numbers and under the same conditions of experiment, are introduced into sea water containing suitable concentrations of dinitrocresol at five-minute intervals throughout the first three divisions, it is found that, regardless of the time of introduction of the eggs into the reagent, about seventy-five per cent are arrested at the commencement or in the early stage of prophase. That this preferential block at the prophase is attributable to the action of the nitro- or halophenol is supported by the observation that, when cell division is blocked by oxygen lack, as for example with KCN, without addition of nitro- or halophenol, the percentage of eggs arrested at the commencement or in the early stage of prophase is substantially smaller than in the series treated with nitro- and halophenols.

Reference has been made previously to the fact that unfertilized sea urchin eggs, when exposed to nitro- and halophenols, exhibit a stimulation of respiration at the same concentrations as do the fertilized eggs, reaching a peak and showing a subsequent fall of respiration at concentrations corresponding with those required to give the same effect in the fertilized eggs. It is interesting to note that, even though the respiration may be maintained at a level three or four times the normal, the unfertilized eggs, when returned to sea water after a period of three or four hours, are readily fertilized by sperm, while, under similar conditions, untreated, mature, unfertilized eggs may have deteriorated to a point at which little or no fertilization occurs. In other words, the treatment with nitrophenol, which has resulted in establishment of a high rate of respiration and a substantial waste of energy, has in a sense preserved the eggs and has not in any way impaired their capacity for fertilization.

When mature, unfertilized sea urchin eggs are exposed to nitro- and halophenols over the same range of concentrations at which respiration is stimulated and then inhibited, and cell division is reversibly blocked in fertilized eggs, a similar respiration curve is observed, with a peak of oxygen consumption several times the normal at approximately the same concentration at which a peak is observed with the fertilized eggs. It is very interesting to note that such unfertilized eggs, held in nitrophenol solutions at concentrations at which respiration is maintained at three or four times the normal for three to six hours, when returned to sea water and exposed to sperm, show a higher percentage of fertilization than do the control eggs, which apparently tend to deteriorate more rapidly on standing in sea water than do the treated eggs.

Nitro- and halophenols do not appear to exert any effect on isolated and purified enzymes. They may, however, be used to suppress such enzymatic effects in living cells, presumably by interfering with the formation of the enzymes in question, rather than by blocking their action once they are formed. This may be accomplished without permanent injury to the cells themselves, the whole mechanism being freely reversible.

For example, the therapeutic efficacy of various penicillins, particularly

penicillin K, is seriously impaired as a result of the penicillin being taken up in large amounts by the tissues of the body, particularly the liver, and subsequently destroyed. This effect may be demonstrated in the Warburg apparatus by using slices of rat liver in contact with appropriate aqueous solutions containing various concentrations of given penicillins.

It has already been reported that sodium azide and certain nitrophenols tend to suppress the destruction of penicillin in the tissue cells.[26] It has now been found that, with increasing concentrations of various nitrophenols under aerobic conditions, with falling oxygen consumption, the destruction of penicillin by the tissue enzymes gradually falls, until a point is reached at which the amount of penicillin remaining in the aqueous phase is equal to that originally introduced, less the amount actually dissolved or taken up at any one time in the liver slices employed. Similarly, under anaerobic conditions, the nitrophenol blocks the destruction of penicillin to an extent fully equal to that exhibited in the aerobic system. From the accompanying charts (FIGURES 8 and 9), the oxygen consumption under anaerobic conditions and the penicillin loss under both aerobic and anaerobic conditions, under the influence of rising concentrations of 4,6-dinitro-o-cresol, 4,6-dinitrocarvacrol, and 4,6-dinitrothymol, indicate clearly that these reagents tend to block the destruction of penicillin by a tissue enzyme until, finally, the penicillin loss from the aqueous phase is only the amount which may normally be taken up by the tissues. This was determined by means of control experiments using, in the Warburg apparatus, tissue slices which had previously been subjected to treatment at a temperature at which the enzymes in question were found to be destroyed.

The fact that, in all cases, the falling respiratory curves conform closely with the falling curves of penicillin destruction, in a range of nitrophenol concentrations which are known to inhibit phosphorylation, supports the conclusion that the protection of penicillin from destruction by the tissues is effected by preventing the synthesis of the enzymes causing destruction, rather than by blocking the action of the enzyme once it is formed. This harmonizes with the observation that nitrophenols exert no inhibiting effect on the destruction of penicillin by isolated enzymes capable of causing such destruction. This block to the destruction of penicillin, exerted by nitrophenols, is apparently freely reversible, since the tissue slices, when washed free of nitrophenol in an appropriate medium, once more exhibit a capacity to destroy penicillin at a rate not far below that normally exhibited. As might be expected, the nitrophenol was found to exert no effect whatever on the gradual destruction or loss of penicillin in an aqueous solution.

It is obvious that in order to clarify further the problems of respiration and cell division block discussed in this review, the first requisite is to isolate, from sea urchin eggs, yeast, and other organisms, cell-free cyclophorase systems similar to those used by Green, Loomis, and Auerbach and Loomis and Lipmann. With such systems available, the problems of penetration into, and distribution through, the cell could be eliminated. Experiments could be conducted over a wide range of concentrations of nitro- and halophenols and phosphate, at varying pH, temperatures, oxygen, CO_2 tension,

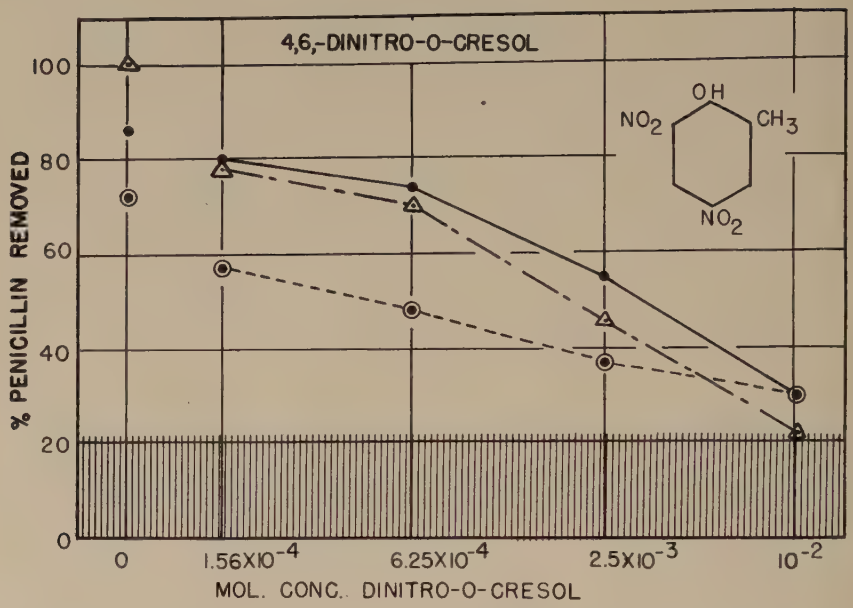


FIGURE 8. Comparative per cent penicillin K removed when solutions containing 160 units per cc. were exposed in the Warburg apparatus at pH 6 and 38° C. for three hours to the action of 160 mg. liver slices per cc. with varying concentrations of 4,6-dinitro-o-cresol under anaerobic and aerobic conditions, the latter with oxygen consumption. Per cent removed under: anaerobic conditions ●——●; aerobic conditions ⊙ - - - - - ⊙. Oxygen consumed in treated slices × 100/oxygen consumed in control slices △—·—△. Shaded area represents amount of penicillin taken up and remaining unchanged in 160 mg. per cc. of liver slices preheated to 65° C for 30 minutes to destroy enzyme action.

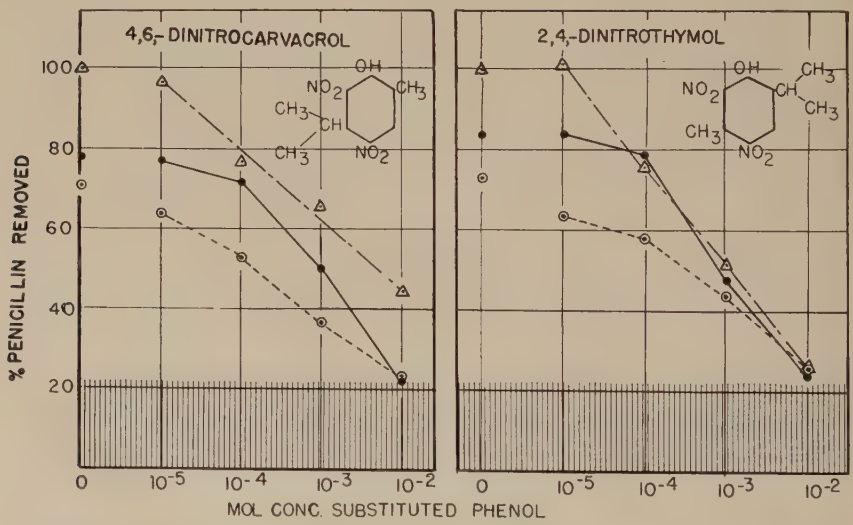


FIGURE 9. Comparative per cent penicillin K removed when solutions containing 160 units per cc. were exposed in the Warburg apparatus at pH 6 and 38° C. for three hours to the action of 160 mg. liver slices per cc. with varying concentrations of 4,6-dinitrocarvacrol and 2,4-dinitrothymol under anaerobic and aerobic conditions, the latter with oxygen consumption. Per cent removed under: anaerobic conditions ●——●; aerobic conditions ⊙ - - - - - ⊙. Oxygen consumed in treated slices × 100/oxygen consumed in control slices △—·—△. Shaded area represents amount of penicillin taken up and remaining unchanged in 160 mg. per cc. of liver slices preheated to 65° C. for 30 minutes to destroy enzyme action.

etc. With such a system, it should be possible to throw light on many of the problems considered in this review, particularly those relating to the interrelation between respiration and cell division. A correlation of data obtainable with these cell-free phosphorylating systems with those already available regarding inhibition of cell division may ultimately serve to throw light on the mechanism of cell division, both normal and pathological.*

Bibliography

1. Krahl, M. E. & G. H. A. Clowes. 1934. Action of dinitrocresol on respiration and cell division in *Arbacia* eggs. Biol. Bull. **67:** 332.
2. Clowes, G. H. A. & M. E. Krahl. 1934. Action of dinitro compounds on sea urchin eggs. Preliminary. Science **80:** 384.
3. Clowes, G. H. A. & M. E. Krahl. 1934. Action of certain oxidative stimulants and depressants on respiration and cell division. Lilly Dedication Volume, October 12.
4. Clowes, G. H. A. & M. E. Krahl. 1936. Studies on cell metabolism and cell division. I. On the relation between molecular structures, chemical properties, and biological activities of the nitrophenols. J. Gen. Physiol. **20:** 145.
5. Krahl, M. E. & G. H. A. Clowes. 1936. Studies on cell metabolism and cell division. II. Stimulation of cellular oxidation and reversible inhibition of cell division by dihalo and trihalophenols. J. Gen. Physiol. **20:** 173.
6. Krahl, M. E. & G. H. A. Clowes. 1935. Some effects of dinitrocresol on oxidation and fermentation. J. Biol. Chem. **111:** 355.
7. Martin, A. W. & J. Field, 2nd. 1934. Inhibition of yeast growth by 2-4 dinitrophenol. Proc. Soc. Exper. Biol. & Med. **32:** 54.
8. Krahl, M. E., A. K. Keltch, & G. H. A. Clowes. 1940. Inhibition of flavoprotein oxidative catalysis by substituted phenols. J. Biol. Chem. **136:** 563.
9. Haas, E., C. J. Harrer, & T. R. Hogness. 1942. Cytochrome reductase. II. Improved method of isolation; inhibition and inactivation; reaction with oxygen. J. Biol. Chem. **143:** 341.
10. Hotchkiss, R. D. 1944. Gramicidin, tyrocidine and tyrothricin. Advances in Enzymology and Related Subjects. By F. F. Nord and C. H. Werkman. Interscience Publishers. New York, N. Y. **4:** 153.
11. Winzler, R. J., D. Burk, & V. du Vigneaud. 1944. Biotin in fermentation, respiration, growth and nitrogen assimilation by yeast. Arch. Biochem. **5:** 25.
12. Clifton, C. E. 1946. Microbial assimilations. Advances in Enzymology and Related Subjects. By F. F. Nord and C. H. Werkman. Interscience Publishers. New York, N. Y. **6:** 269.
13. Clowes, G. H. A., M. E. Krahl, & Anna K. Keltch. 1937. Substituted phenols as inhibitants of the fertilization of *Arbacia* and of ciliary movement of *Arenicola* larvae. Biol. Bull. **73:** 376.
14. Loomis, W. F. & F. Lipmann. 1948. Reversible inhibition of the coupling between phosphorylation and oxidation. J. Biol. Chem. **173:** 807.
15. Green, D. E., W. F. Loomis, & V. H. Auerbach. 1948. Studies on the cyclophorase system. I. The complete oxidation of pyruvic acid to carbon dioxide and water. J. Biol. Chem. **172:** 386.
16. Clowes, G. H. A. & M. E. Krahl. 1936. Antagonistic action of dinitrothymol and dinitrocresol on cellular respiration. Proc. Soc. Exper. Biol. and Med. **34:** 565.
17. Smith, H. W. & G. H. A. Clowes. 1924. The influence of carbon dioxide on the velocity of division of marine eggs. Am. J. Physiol. **68:** 183.
18. Krahl, M. E. & G. H. A. Clowes. 1938. Physiological effects of nitro- and halo-substituted phenols in relation to extracellular and intracellular hydrogen ion concentration. I. Dissociation constants and theory. J. Cell. and Comp. Physiol. **11:** 1.
19. Clowes, G. H. A. 1942. Interactions of biologically significant substances in surface films. Symposium on surface chemistry, Fiftieth Anniversary, University of Chicago, September, 1941. Publication No. 21: 1–16. American Association for Advancement of Science.
20. Clowes, G. H. A. & M. E. Krahl. 1940. Studies on cell metabolism and cell division. III. Oxygen consumption and cell division of fertilized sea urchin eggs in the presence of respiratory inhibitants. J. Gen. Physiol. **23:** 401.

* See addenda following bibliography.

21. Krahl, M. E. & G. H. A. Clowes. 1940. Studies on cell metabolism and cell division. IV. Combined action of substituted phenols, cyanide, carbon monoxide, and other respiratory inhibitors on respiration and cell division. J. Gen. Physiol. **23:** 413.
22. Krahl, M. E. & G. H. A. Clowes. 1938. Physiological effects of nitro- and halo-substituted phenols in relation to extracellular and intracellular hydrogen ion concentration. II. Experiments with *Arbacia* eggs. J. Cell. & Comp. Physiol. **11:** 21.
23. Hutchens, J. O., M. E. Krahl, & G. H. A. Clowes. 1939. Physiological effects of nitro- and halo- substituted phenols on *Arbacia* eggs in the presence of ammonia. J. Cell. & Comp. Physiol. **14:** 313.
24. Clowes, G. H. A., A. K. Keltch, & M. E. Krahl. 1940. Extracellular and intracellular hydrogen ion concentration in relation to anesthetic effects of barbituric acid derivatives. J. Pharm. & Exper. Therap. **68:** 312.
25. Krahl, M. E., A. K. Keltch, & G. H. A. Clowes. 1940. The role of changes in extracellular and intracellular hydrogen ion concentration in the action of local anesthetic bases. J. Pharm. & Exper. Therap. **68:** 330.
26. Clowes, G. H. A. & A. K. Keltch. 1947. Supplementary report on the comparative results obtained with crystalline penicillins, particularly G and K, when exposed in the Warburg apparatus to the action of normal and preheated rat liver slices, homogenized liver, and bacteria; also normal liver with enzyme-suppressing chemical agents. Presented at Conference on Antibiotic Research, Washington, D. C., January 31 and February 1, 1947, under auspices of Antibiotics Study Section of National Institute of Health.

*Addenda**

Since the completion of this paper, cell-free, oxidative, phosphorylating systems have been obtained from both unfertilized and fertilized *Arbacia* eggs. When these systems are treated with a wide range of concentrations of nitro- and halophenols, the stimulation and subsequent suppression of respiration and the reversible suppression of phosphorylation correspond with the above-reported stimulation and suppression of respiration and the reversible block to cell division of normal fertilized *Arbacia* eggs and are obtained at corresponding concentrations. The isomers, dinitrocarvacrol and dinitrothymol, both of which give a reversible suppression of cell division, also give a reversible suppression of phosphorylation at corresponding concentrations. Dinitrocarvacrol, which stimulates the respiration of the intact eggs, also stimulates the respiration of the phosphorylating system, while dinitrothymol inhibits the respiration of both the fertilized eggs and the phosphorylating system through the entire range of concentrations of the reagent. Those nitro- and halophenols, which exert no effect on the respiration and cell division of fertilized *Arbacia* eggs, exert no effect on the respiration and phosphorylation of the cell-free, oxidative, phosphorylating systems obtained from the eggs. These series of experiments indicate that the effects exerted on the intact eggs are attributable to the effects exerted on the interiorly contained, oxidative, phosphorylating systems. Experiments conducted on the eggs of *Mactra solidissima* gave results paralleling those obtained with *Arbacia*, but substantially higher concentrations of the nitro- and halophenols were required to give respiration peaks and reversible blocks to cell division.

* Added by the author December 1, 1950, at time of proof correction.

Bibliography

Crane, R. K. & A. K. Keltch. 1949. Dinitrocresol and phosphate stimulation of the oxygen consumption of a cell-free oxidative system obtained from sea urchin eggs. J. Exper. Med. **32:** 503.

Keltch, A. K., C. F. Strittmatter, C. P. Walters, & G. H. A. Clowes. 1950. Oxidative phosphorylation by a cell-free particulate system from unfertilized *Arbacia* eggs. J. Gen. Physiol. **33:** 547.

Clowes, G. H. A., A. K. Keltch, C. F. Strittmatter, & C. P. Walters. 1950. Action of nitro- and halophenols upon oxygen consumption and phosphorylation by a cell-free particulate system from *Arbacia* eggs. J. Gen. Physiol. **33:** 555.

Strittmatter, P., A. K. Keltch, C. P. Walters, & G. H. A. Clowes. 1950. Oxidative phosphorylation by a cell-free, particulate, enzyme system derived from fertilized *Arbacia* eggs. Biol. Bull. **99:** 333.

Keltch, A. K., P. Strittmatter, C. P. Walters, & G. H. A. Clowes. 1950. Action of a series of nitro- and halophenols on the respiration and cell division of *Mactra solidissima*. Biol. Bull. **99:** 334.

Clowes, G. H. A., A. K. Keltch, P. Strittmatter, & C. P. Walters. 1950. Action of dinitrocarvacrol and dinitrothymol on respiration of fertilized *Arbacia* and *Mactra* eggs and certain mammalian tissues and cell-free, particulate, phosphorylating systems. Biol. Bull. **99:** 335.

THE ACTION ON NITROGEN MUSTARDS AND RELATED SUBSTANCES ON CELL DIVISION

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I. Chemical and Biochemical Reactions of Mustard

The chemical formula of mustard gas is shown in FIGURE 1. Some analogues that possess similar biological and chemical reactivities are shown in FIGURES 2 and 3. The latter is the general formula for the nitrogen mustards. Substances of this type have proved particularly useful in biological experimentation since, by varying the group R, it is possible to vary the physico-chemical characteristics of the molecule and thus obtain an extended group of substances which vary somewhat in their penetrating power, reactions, rates, *etc.* Much of the current work is based on the hope that, by a suitable variation in the R group, substances will be obtained that have a higher degree of biological selectivity than the simplest compounds in this series.

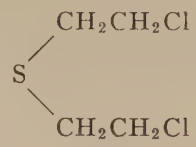

FIGURE 1

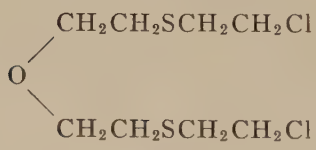

FIGURE 2

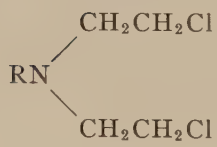

FIGURE 3

The betachlorethyl groups in the mustards give these compounds their high reactivity. In a nonaqueous environment, these groups are quite stable, but in the presence of water an activation occurs. In the case of the nitrogen mustards, the nature of the activation reaction is known in some detail, particularly from the studies of Bergmann, Fruton, and Golumbic,[17, 18, 19, 21, 22, 23, 50] and of Cohen and his associates.[7] These investigators have shown that, in the presence of water, one of the betachlorethyl

groups loses its chlorine and is converted into an ethylene imine, the whole molecule thus becoming an ethylene imonium compound (FIGURE 4).

This reaction does not occur if the original compound is in the form of an ammonium salt. The activation of the nitrogen mustards is, therefore, inhibited at low pH, and stable aqueous solutions of these compounds in the presence of M/1000 HCl can be prepared. It is presumed that the sulfur mustards also undergo activation through the formation of an intermediate ethylene sulfonium compound, but, if this is true, the latter is so unstable that its presence has not as yet been demonstrable.

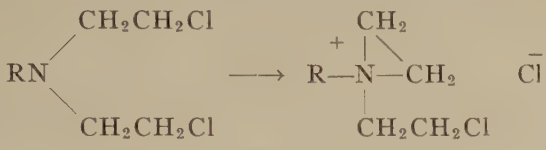

FIGURE 4

The ethylene imonium compounds and, presumably, the sulfonium analogues are highly reactive substances, and, on reaction, the three membered ring is opened and the ethylene group attached at one end to whatever compound it reacts with. In the unionized tertiary amine that results, the second betaethylchloride can then be activated, and a second reactive ethylene imonium structure results. In general, the rate of activation of the second betaethylchloride is slower than the first, and the toxicity of these agents is much less in the second stage of their reactivity than in their first stage. It is possible, of course, to construct mustard analogues with only a single betachlorethyl group, and many of those engaged in this field have felt that a more extensive exploration of the chemical reactivities and toxic effects of these "one-arm mustards" would be of great value. During the war work on these agents, Dr. du Vigneaud was particularly emphatic concerning the advantages which such simplification of the problem could bring. A number of one-arm mustards were synthesized in his department, and these substances were fruitfully utilized in analyzing the chemical reactivity of this configuration.

Among the one-arm nitrogen mustards, the one most extensively studied is "dibenamine" (FIGURE 5), which was shown by Nickerson and Goodman[48] to have remarkable sympatholytic properties, *i.e.*, it abolishes the excitatory responses to adrenalin, but does not affect the inhibitory actions of adrenalin. This compound and many of its analogues have been studied from the point of view of their general pharmacology. They are locally irritant but relatively nontoxic, and, in effectively sympatholytic doses, do not produce leukopenia. Unfortunately, their effect on mitosis has not been studied and a wide area in this field remains to be explored. These one-arm mustards are, of course, very much less toxic than the two-arm analogues, but the wide variety of groups that might be attached to such molecules greatly enhances the field of their possible selective biological action. An example of what might be done with such agents is the following. Up to the present, we have little knowledge of the cytological location of the mustard reaction.

A one-arm mustard so constructed as to yield a histochemically recognizable reaction in its attached group could greatly assist in discovering the site of reacted mustard. A project to work toward this goal was considered during the war but abandoned when the emergency, in respect to gas warfare, diminished.

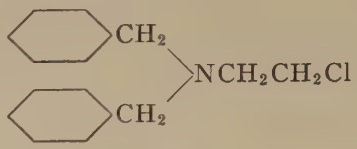

FIGURE 5

The activated derivatives of the mustards are capable of reacting with a great variety of agents (FIGURE 6). They react with water to form alcohols (1), with alcohols to form ethers (2), with sulfhydryl compounds to form thioethers (3), with amines to form secondary amines (4), and with carboxylic acids and some inorganic acids to form esters (5, 6). In addition, these substances react with thioethers to form sulfonium compounds.

(1) $RCH_2CH_2Cl + H_2O \longrightarrow RCH_2CH_2OH + HCl$

(2) $RCH_2CH_2Cl + HOR_1 \longrightarrow RCH_2CH_2OR_1 + HCl$

(3) $RCH_2CH_2Cl + HSR_1 \longrightarrow RCH_2CH_2SR_1 + HCl$

(4) $RCH_2CH_2Cl + H_2NR_1 \longrightarrow RCH_2CH_2NHR_1 + HCl$

(5) $RCH_2CH_2Cl + HOOCR_1 \longrightarrow RCH_2CH_2OCOR_1 + HCl$

(6) $RCH_2CH_2Cl + H_3PO_4 \longrightarrow RCH_2CH_2OPO_3H_2 + HCl$

FIGURE 6

Reactions of this type have been observed between mustard and methionine (FIGURE 7), between mustard and cysteine (FIGURE 8), and between mustard and its own hydrolysis product, thiodyglycol (FIGURE 9). Analogous reactions with secondary and tertiary amines to form tertiary and quarternary products also occur. In particular, the nitrogen mustards can react with themselves to form double molecules (FIGURE 10) or even more complicated polymers.

In an homogeneous aqueous solution, the various reactive substances present compete among themselves for reaction with mustard in proportion to their concentrations and their reactivities. The latter is generally expressed as the competition factor of the particular substance, and extensive studies have made it possible to assign numerical values of this competition factor to a variety of substances in relation to each other or in relation to water as a chosen standard. Among the biochemical substances potentially capable of reacting with mustard, the sulfhydryl compounds have by far the highest competition factors, and it has been assumed by many that

the biological effects of mustard are to be attributed chiefly to the alkylation of particular sulfhydryl groups and the consequent inactivation of particular sulfhydryl enzymes.

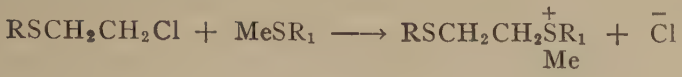

FIGURE 7

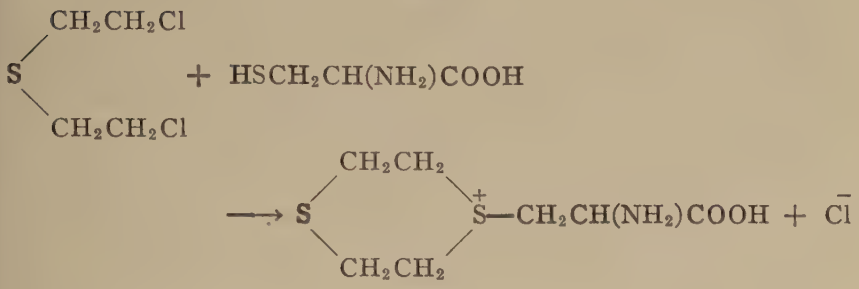

FIGURE 8

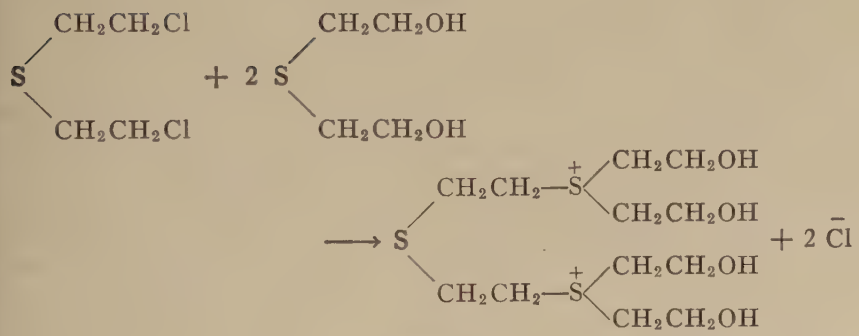

FIGURE 9

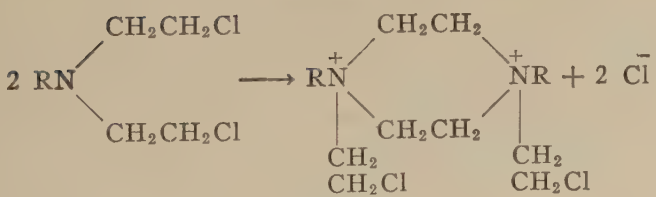

FIGURE 10

While arguments based on experiments in homogeneous aqueous solutions have some attractive force, their applicability to the nonhomogeneous internal environment of the cell is burdened with large uncertainties. Mustard, and also the nitrogen mustards at physiological pH, are poorly soluble in water and readily soluble in lipoids. Consequently, one would expect

their primary distribution in tissue to be largely in the lipoidal components. Within a lipoidal environment, these substances are unreactive. It has been shown, for instance, that the half-life of mustard in an aqueous lecithin emulsion is many times that in a homogeneous aqueous environment. In order to become activated, the mustard would have to emerge from the lipoid environment. It is quite possible, therefore, that the distribution of activated mustard within the cell may be very inhomogeneous, and that the choice of tissue receptors may be more influenced by their intracellular location than by their competition factors.

Evidence to support this conclusion was obtained in the course of our studies on the corneal epithelium.[11] This tissue contains considerable amounts of glutathione. After very severe exposure to mustard (more than 1,000 times the dose required to produce severe mitosis inhibition sufficient to produce marked inhibition in certain metabolic pathways), we found that less than 5 per cent of the glutathione in the tissue had reacted with mustard. When the same dosage of mustard was applied to homogenized corneal epithelium, approximately 50 per cent of the glutathione reacted with mustard. In homogeneous aqueous solution, glutathione has a competition factor among the highest of biochemical substances. In the structurally intact tissue, it is plain that some substances with very high competition factors are relatively protected from reaction with mustard.

Somewhat related results were obtained by Kinsey and Grant[42, 44] on yeast. Of the organically bound mustard in yeast, they found 10 per cent attached to glutathione, 50 per cent to extractable proteins, of which some were identified as concerned in the carbohydrate metabolism, and 40 per cent to the inextractable structural framework of the cell. They found no evidence indicating the presence of any substance greatly exceeding the sulfhydryl compounds in its capacity to bind mustard. It is to be concluded that not only the reactivity of the cellular components but also their location markedly affects their selection as receptors for mustard. On *a priori* grounds, one would expect, therefore, that substances located near the lipoidal structures of the cell, such as the cell membrane and the nuclear membrane, might be among those most readily damaged by mustard. Some slight histochemical support for this conclusion was obtained on the corneal epithelium, but our knowledge of the nature and location of the intracellular receptors of mustard injury is still in the state of vague guesses. The lipoid solubility of the unreacted mustards and the water solubility of their activated derivatives may contribute important aspects to their penetrating power, as has been pointed out by Gilman and Philips.[49]

II. Biological Effects

1. *Mitosis Inhibition.* Inhibition of mitosis occurs as the lowest threshold phenomenon of mustard injury to cells and tissues. With sufficiently small doses, spontaneous recovery from the inhibition occurs, and no permanent damage has been noted. The effect of mustard and of its analogues in this respect appears to be well nigh universal. It has been observed in the corneal epithelium, the bone marrow, the intestinal epithelium,[9, 12] and the

regenerating liver[46] of mammals. Cannan[36] studied the phenomenon in starfish ova and Kinsey and Grant in yeast.[40, 41, 43, 45] Extensive studies on amphibian embryos have been made by Gillette and Bodenstein.[20] Tenbrock and Herriot[51] reported the inactivation of certain viruses by mustard, but whether this is a recoverable phenomenon at sufficiently low dosage has not as yet been demonstrated.

The inhibition of mitosis does not develop immediately after the reaction of mustard with the tissue, provided the dosage is not excessive. Using a nitrogen mustard whose half-life in the tissue is at most 20 to 30 minutes, we found the inhibition of mitosis in the rat's cornea to reach a maximum in 18 to 24 hours. With the same agent, Bodenstein found the inhibition in amphibian embryos to become maximal two days after exposure. A similar, though shorter, interval between exposure and maximal effect was found by Kinsey and Grant in yeast. It is evident that the effect of mustard on the mitotic mechanism is an indirect one.

With threshold doses, the arrest of mitosis is in the interphase, and the arrest does not interfere with cellular growth[12] and differentiation. In the amphibian embryo, cells of amazingly gigantic size and progressively differentiated characteristics have been described.[4, 5] While, at threshold dosage, the arrest occurs at interphase, with larger doses a slowing down of the progress of cells already in mitosis is observed in the corneal epithelium[12] and in the starfish ovum.[36] This slowing down of the mitotic process apparently affects all of the active phases of mitosis equally. This is in contrast to the differential effect of X-radiation on various phases of the mitotic cycle noted by Carlson and Hollander.[6]

2. *Injury to Proliferating Tissues.* The inhibition of mitosis produced by threshold doses of mustard is spontaneously overcome. With larger doses, irreparable damage occurs, leading to cellular death. There are several reasons for believing that some aspects of this necrotizing effect are related to the mitotic process.

a. Actively proliferating tissues have the highest susceptibility to damage of this type. In the adult mammal, systemic mustard poisoning yields extensive lesions in the bone marrow, lymph nodes, intestinal mucosa, *etc.*, while the neuromuscular system, liver, kidneys, and other organs which are not actively proliferating escape injury, unless the dose is so great as to be immediately fatal.[24, 37, 39] Regenerating liver, after partial hepatectomy, is, however, susceptible to mustard damage.[46] In the embryo, the difference in susceptibility between actively proliferating tissue and non-proliferating tissue in the process of differentiation is so sharp that Gillette and Bodenstein[20] have proposed the use of nitrogen mustards as a tool with which to analyze embryonic growth and differentiation. They found that, in the embryo, cells that are no longer proliferating are uninjured, with the dosage employed, and differentiate normally. Among proliferating cells, two different reactions were noted. In some proliferating regions, after a few days, the cells show fragmentation of the nucleus and disintegrate. In other regions, the proliferating cells are inhibited from dividing, but grow to gigantic size and eventually disintegrate some ten days after exposure. The

explanation of this difference in behavior is obscure. It may be related to the growth capacity of the cells or to a variation in their sensitivity to injury at different stages of differentiation.

b. There is strong evidence that the high susceptibility in some proliferating tissues specifically characterizes the end of the interphase period of the mitotic cycle, the phase that may perhaps be designated most simply as "premitosis." Cannan's studies on starfish ova showed that the highest susceptibility in these cells occurred during the 15 minutes before the onset of prophase. In the corneal epithelium,[13] in which only the basal cell layers are actively proliferating, and in which the interphase is, on the average, about 200 hours, only a very small number of cells in the basal layers normally show high susceptibility to mustard injury. If the onset of mitosis is inhibited by such drugs as adrenalin or morphine, however, cells accumulate in the susceptible state in appreciable numbers.

c. In many tissues, the mode of death of the susceptible cells is characterized by fragmentation of the nucleus. The detailed steps in this process bear some cytological resemblance to the prophase and early metaphase of normal mitosis. In particular, the nuclear fragmentation in the corneal epithelium is accompanied by an increase in the Fuelgen-positive material in the nucleus. This mode of cellular death resembles that resulting from ultraviolet irradiation, and is similar to that seen in the bone marrow and lymph nodes after X radiation.[24, 39]

The high susceptibility of actively proliferating tissue may, therefore, be explained by the high proportion of cells in the premitotic state that characterizes these tissues. It is not to be concluded from this that all cells in premitosis are equally susceptible. The resistance to mustard damage varies from tissue to tissue and from species to species, but, for each of the few cell types so far studied in detail, the maximum susceptibility appears to occur during premitosis.

If we now reverse the argument and assume that high susceptibility to death by nuclear fragmentation, following exposure to mustard, is evidence that the affected cell was in premitosis at the time of exposure, we can define more closely the locus of mitosis inhibition by threshold doses of the mustard agents.[13] A dose may be chosen for the rat's cornea which produced no nuclear fragmentation, but which, nevertheless, completely suppressed all visible mitotic activity 24 hours after its administration. If a second larger dose of mustard is administered during this period of mitosis inhibition, approximately the usual number of basal cells undergo nuclear fragmentation. It is to be concluded that the mitotic inhibition of the threshold dose does not prevent these cells from entering the premitotic phase in their usual numbers, and that the effect of the threshold dose is to prevent a cell from completing the premitotic transformation and entering mitosis. It should be noted, however, that it is not the cells in premitosis at the time of exposure to mustard that are arrested in their progress throught the mitotic cycle. The maximum inhibition of mitosis is seen many hours after exposure. Consequently, we have to conclude that the damage occurs to cells that are in an earlier stage of interphase, and that

the damage is such that, though these cells are still able to reach premitosis, they are not then able to progress further in the mitotic cycle.

Since death by nuclear fragmentation occurs after many different types of injury, and even is seen occasionally in normal control tissues, it is reasonable to suppose that the premitotic state is an essentially unstable one. There is some evidence that the premitotic state is a reversible one. The simplest explanation that we have found to account for all of these findings is that threshold doses of mustard agents affect cells that subsequently arrive in premitosis so that they are unable to progress to the active phase of the mitotic cycle, while larger doses render cells in premitosis unable to return to the resting phase. Cells frozen in the unstable premitotic state may then be supposed to die by passing into an incomplete and pathological mitosis. This hypothesis is merely that which appears to be the simplest explanation of the available facts at the moment, and it is presented with due reserve.

Karnofsky[38] has found that animals first treated with fairly large doses of mustard are thereby rendered more susceptible to subsequent treatment with X radiation, but that animals first treated with X radiation are not, thereby, rendered more susceptible to subsequent treatment with mustard. It may be worth while to ask whether cells in premitosis are particularly susceptible to damage by X radiation, and whether further experiments along the line initiated by Karnofsky may provide a suitable test for the hypotheses of the possible freezing of cells in premitosis following exposures to mustard suggested above.

The suppression of mitotic activity in amphibian larvae has been shown by Bodenstein[5] to be associated with a suppression of the synthesis of desoxyribose nucleic acid, the synthesis of ribose nucleic acid being unimpaired. Bodenstein has suggested, therefore, that mitosis is inhibited because the cell cannot synthesize desoxyribose nucleic acid. The validity of this conclusion is somewhat doubtful, since desoxyribose nucleic acid synthesis normally occurs mainly during mitosis. If mitosis were inhibited by any mechanism, the synthesis of desoxyribose nucleic acid would necessarily be greatly reduced. It is interesting that Hevesy[34] has found that, during the inhibition of mitosis by X radiation, the turnover of phosphate in desoxyribose nucleic acid is suppressed. In this case, there is also no evidence that the primary damage by X radiation is done to the nucleic acid transphosphorylase.

3. *Loss of Cellular Cohesion and Metabolic Inhibitions.* The mustard agents, when applied locally to the skin, produce vesication. Danielli[8] has shown that this phenomenon depends in part on a loss of cohesion between adjacent cellular layers, and Herrmann and Hickman[25–27] have found a similar loss of cohesion between the corneal epithelium and stroma after exposure to mustard. The threshold dosage in the cornea which produces this loosening is also the threshold at which metabolic inhibitions are first recognizable.[28–33] The metabolic effects that are found in the cornea with this level of dosage primarily concern the inhibition of certain metabolic interactions that normally occur between the epithelium and stroma in the

intact organized corneal tissue.[10, 16] There is no suggestion of any link between these metabolic and vesicant effects of mustard and its effect on the mitotic mechanism. These vesication phenomena apparently result from damage by mustard to cellular elements that lie at or near certain portions of the cell boundary. They are mentioned here merely to raise the question as to whether, in the normal cell, any metabolic interaction between nucleus and cytoplasm may occur, whether the pattern of such interaction may not undergo a cycle of changes during the mitotic cycle, and whether the mitotic effects of mustard may be due to damage to the mechanism of such possible interaction.

4. *Necrosis of Nonproliferating Tissues.* With sufficiently large dosages of mustard agents, necrosis is seen also in nonproliferating tissues. The affected cells, for the most part, die by pyknosis,[47] a process so nonspecific and so poorly understood that it sheds no light on the mitotic mechanism. In some special tissues, the death of the nonproliferating cells is associated with a swelling and bursting of their nuclei.[14]

5. *Mutations Induced by Mustard.* The production of mutations in *Tradescantia* and *Drosophila* has been reported by Auerbach and Robson,[1, 2, 3] in *Neurospora* by Horowitz, Houlahan, Hungate, and Wright.[35] According to Auerbach, some of the mutations produced in *Drosophila* are characterized by abnormal segregation of the chromosomes in meiosis. While the production of mutations is surely to be classified as a mitotic effect, it seems to me that the chemical reactions concerned in mutagenesis must be so highly localized in particular chromosomal elements as to throw little light on the general mechanism of mitosis.

III. Discussion

The Conference Chairman has asked that the contributions to this monograph be directed primarily toward the analysis of the mitotic mechanism. Unfortunately, the multiplicity of possible chemical reactions between mustard and various tissue components leaves large uncertainties as to the nature and location of the primary mustard injury. When the multiplicity of possible receptors for mustard is permuted with the multiplicity of its effects at various dosage levels and with the multiplicity of unknowns regarding the mitotic mechanism, very few conclusions can be expected to emerge. This is not to disparage the utility of mustard as a tool for the study of mitosis, but merely to emphasize the complexity of the mitotic process and the extent of our ignorance about it. Obstacles of a similar magnitude confront us in the use of ultraviolet or X radiation for the study of the mitotic mechanism. Since these three agents have many similiarities and some differences in their effects, the detailed comparison of their action on the same test objects[15] and the study of the effects of combined or successive applications[38] may sharpen our interpretations.

Bibliography

1. Auerbach, C. & J. M. Robson. 1946. Mutation from mustard and related substances. Nature **157:** 302.
2. Auerbach, C. 1947. Abnormal segregation after chemical treatment of *Drosophila*. Genetics **32:** 3–7.

3. Auerbach, C., J. M. Robson, & J. G. Carr. 1947. Chemical production of mutations. Science **105:** 243–7.
4. Bodenstein, D. 1947. The effects of nitrogen mustard on embryonic amphibian development. I. Ecdodermal effects. J. Exp. Zool. **104** (3): 311–342.
5. Bodenstein, D. & A. A. Kondritzer. 1948. The effect of nitrogen mustard on nucleic acids during embryonic amphibian development. J. Exper. Zool. **107** (1): 109–122.
6. Carlson, J. G. & A. Hollaender. 1944. Immediate effects of low doses of ultraviolet radiation of wavelength 2537 Å on mitosis in the grasshopper neuroblast. J. Cell. & Comp. Physiol. **23:** 157–160.
7. Cohen, B., J. Harris, E. R. Van Artsdalen, & M. E. Perkins. 1948. Reaction kinetics of aliphatic tertiary β-chloroethylamines in dilute aqueous solution. I. The cyclization process. J. Amer. Chem. Soc. **70:** 281.
8. Danielli, J. F. & M. Danielli. Unpublished.
9. Friedenwald, J. S., W. Buschke, R. O. Scholz, & S. G. Moses. 1947. Some effect of sulfur and nitrogen mustards on cell nuclei in mammalian cornea. Nitrogen Mustards. Amer. Asso. for Advancement of Science, Amer. Cancer Soc.
10. Friedenwald, J. S. & A. C. Woods. 1948. Studies on the physiology, biochemistry, and cytopathology of the cornea in relation to injury by mustard gas and allied toxic agents. I. Introduction and Outline. Bull. Johns Hopkins Hosp. **82** (2): 81.
11. Friedenwald, J. S., W. Buschke, R. O. Scholz, A. Snell, Jr., & S. G. Moses. 1948. II. Primary reaction of mustard with the corneal epithelium. *Ibid*: 102.
12. Friedenwald, J. S., W. Buschke, & R. O. Scholz. 1948. IV. Effects of mustard and nitrogen mustard on mitotic and wound healing activities of the corneal epithelium. *Ibid*: 148.
13. Friedenwald, J. S. & W. Buschke. 1948. V. Nuclear fragmentation produced by mustard and nitrogen mustards in the corneal epithelium. *Ibid*: 161.
14. Friedenwald, J. S. 1948. VI. Note on karyolysis of the corneal stroma cells. *Ibid*: 178.
15. Friedenwald, J. S., W. Buschke, & S. G. Moses. 1948. XVI. Comparison of the effects of mustard, ultraviolet and X-radiation, and colchicine on the cornea. *Ibid*: 326.
16. Friedenwald, J. S. 1948. Studies on the physiology, biochemistry and cytopathology of the cornea in relation to injury by mustard gas and allied toxic agents. XVII. Summary and some possible interpretations. *Ibid*: 326.
17. Fruton, J. S. & M. Bergmann. 1946. Chemical reactions of the nitrogen mustard gases. III. The transformation of methylbis (β-chloroethyl) amine in water. J. Organ. Chem. **11:** 543–9.
18. Fruton, J. S., W. H. Stein, & M. Bergmann. 1946. V. The reactions of the nitrogen mustard gases with protein constituents. *Ibid*: 559–70.
19. Fruton, J. S., W. H. Stein, M. A. Stahlmann, & C. Golumbic. 1946. VI. The reactions of the nitrogen mustard gases with compounds of biological interest. *Ibid*: 571–80.
20. Gillette, R. & D. Bodenstein. 1946. Specific developmental inhibitions produced in amphibian embryos by a nitrogen mustard compound. J. Exp. Zool. **103** (1): 1–32.
21. Columbic, C., J. S. Fruton & M. Bergmann. 1946. Chemical reactions of the nitrogen mustard gases. I. The transformation of methylbis (β-chloroethyl) amine in water. J. Organ. Chem. **11:** 518–535; II. The composition of aged unbuffered solutions of methyl-bis (β-chloroethyl) amine. *Ibid*: 536–42.
22. Golumbic, C., M. A. Stahlmann, & M. Bergmann. 1946. The transformation of tris (β-chloroethyl) amine in water. *Ibid*: 550–8.
23. Golumbic, C., J. S., Fruton, & M. Bergmann. 1946. VII. Monosubstitution products of ethyl-bis (β-chloroethyl) amine and methyl-bis (β-chloroethyl) amine. *Ibid*: 581–5.
24. Graef, I., D. B. Karnofsky, V. B. Jager, B. Krichesky, & H. W. Smith. 1948. The clinical and pathologic effects of the nitrogen and sulfur mustards in laboratory animals. Am. J. Path. **24:** 1–47.
25. Herrmann, H. & F. H. Hickman. 1948. VII. The adhesion of epithelium to stroma in the cornea. Bull. of Johns Hopkins Hosp. **82** (2): 182.
26. Herrmann, H. 1948. VIII. The effect of histamine and related substances on the cohesion of the corneal epithelium. *Ibid*: 208.
27. Hermann, H. & H. Hickman. 1948. IX. Loosening of the corneal epithelium after exposure to mustard. *Ibid*: 213.
28. Hermann, H. & H. Hickman. X. Exploratory studies on corneal metabolism. *Ibid*: 225.

29. Hermann, H. & H. Hickman. 1948. XI. The effect of mustard on some metabolic processes in the cornea. *Ibid*: 251.
30. Hermann, H. & H. Hickman. 1948. XII. Further experiments on corneal metabolism in respect to glucose and lactic acid. *Ibid*: 260.
31. Hermann, H. & H. Hickman. XIII. The consumption of pyruvate, acetoin, acetate, and butyrate by the cornea. *Ibid*: 273.
32. Hermann, H. & H. Hickman. 1948. XIV. The utilization of ribose and other pentoses by the cornea. *Ibid*: 287.
33. Herrmann, H. & S. G. Moses. 1948. XV. Studies on non-protein nitrogen in the cornea. *Ibid*: 295.
34. Hevesy, G. 1945. On the effect of Roentgen rays on cellular division. Rev. Mod. Phy. **17:** 102.
35. Horowitz, N. H., M. B. Houlahan, M. B. Hungate, & B. Wright. 1946. Mustard gas mutation in *Neurospora*. Science **104:** 333–4.
36. Cannan, R. K. 1944. Personal communication.
37. Karnofsky, D. A., I. Graef, & H. W. Smith. 1948. Studies on the mechanism of action of the nitrogen and sulfur mustards *in vivo*. Am. J. Path. **24:** 275–304.
38. Karnofsky, D. A., J. H. Burchenal, R. A. Ormsbee, I. Cornman, & C. P. Rhoads. 1947. Experimental observations on the use of the nitrogen mustards in the treatment of neoplastic disease. Approaches to Tumor Chemotherapy. Amer. Assoc. for the Advancement of Science, Wash. Also Cancer Research **I:** 1–50, 1947.
39. Kindred, J. E. 1947. Histologic changes occurring in the hemopoietic organs of albino rats after single injections of 2-chloroethyl vesicants. Arch. Path. **43:** 253–295.
40. Kinsey, V. E. & W. M. Grant. 1947. Action of mustard gas and other poisons on yeast cells. I. Effect of mustard gas on the rate of cell divison. J. Cell. & Comp. Physiol. **29** (1): 51–64.
41. Kinsey, V. E. & W. M. Grant. 1947. II. Effect of mustard gas on the mortality, morphology, carbohydrate metabolism, and permeability. *Ibid*: 65–74.
42. Kinsey, V. E. & W. M. Grant. 1947. III. Distribution of fixed mustard gas in yeast. *Ibid*: 75–84.
43. Kinsey, V. E. & W. M. Grant. 1947. IV. Study of the effects of divinyl sulfone and their reversal. J. Cell. & Comp. Physiol. **29** (2): 95–108.
44. Kinsey, V. E. & W. M. Grant. 1947. V. Correlation between the quantity of glutathione bound by mustard and divinyl sulfone and their effect on growth rate. J. Cell & Comp. Physiol. **29** (3): 289–300.
45. Kinsey, V. E. & W. M. Grant. 1947. VI. Study of relationship between inhibition of growth by various poisons, and effects of other toxic agents on yeast. J. Cell. & Comp. Physiol. **30** (1): 31–42.
46. Marshak, A. 1946. Effect of mustard gas on mitosis and P^{32} uptake in regenerating liver. Proc. Soc. Exper. Biol. & Med. **63:** 118–120.
47. Maumenee, A. E. & R. O. Scholz. 1948. Studies on the physiology, biochemistry and cytopathology of the cornea in relation to injury by mustard gas and allied toxic agents. III. The histopathology of the ocular lesions produced by the sulfur and nitrogen mustards. Bull. Johns Hopkins Hosp. **82** (2): 121.
48. Nickerson, M., G. Nomaguchi, & L. S. Goodman. 1946. Relation of structure to activity in a new series of sympatholytic agents. Fed. Proc. **5:** 195; 1945. Pharmacology of series of new sympatholytic agents. Proc. Am. Fed. Clin. Research **2:** 109–110.
49. Philips, F. S. & A. Gilman. 1947. The relation between chemical constitution and biological action of the nitrogen mustards. Nitrogen Mustards. Amer. Assoc. for Advancement of Science. Approaches to Tumor Chemotherapy. National Research Council.
50. Stahlmann, M. A. & M. Bergmann. 1946. Chemical reactions of the nitrogen mustard gases. VIII. The oxidation of nitrogen mustard gases by percaids. J. Organ. Chem. **11:** 586–91.
51. Tenbrock, C. & R. M. Herriott. 1946. Virus inactivation with mustard and use for vaccine. Proc. Soc. Exper. Biol. & Med. **62:** 271–2.

THE ACTION OF PODOPHYLLIN AND ITS FRACTIONS ON MARINE EGGS*

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Background

Podophyllum peltatum is called the Mandrake by those of classical leanings, or May-apple by those whose interest centers on its sweet, yellow fruit. The European mandrake (*Mandragora:* Solanaceae) has for centuries been held of value for certain delicate mythical properties[38] and for very real toxic and narcotic potency. In the New World, the common name was applied to an entirely different herb in a different family (Berberidaceae).

Modern interest in *Podophyllum* has taken a new trend. This stems directly or indirectly from a publication of Kaplan,[23] in which he revealed that podophyllin is strikingly effective in reducing venereal warts, condylomata acuminata, with 25 per cent podophyllin in oil. This simple and effective therapy met with enthusiastic acceptance in both hemispheres, judging from the literature that followed its introduction.[44]

King and Maurice Sullivan,[25] by sectioning biopsy material, showed that podophyllin attacks mitoses in both the condylomata and in normal skin. They were not prepared to say that this destruction of dividing cells is the mechanism of the cure, inasmuch as there is, in addition, a widespread degeneration of a nonspecific character.[36] Common warts did not yield to 25 per cent podophyllin in oil, but 20 per cent podophyllin in 95 per cent alcohol destroyed 15 of a hundred verrucae vulgares.[36]

B. J. Sullivan and Wechsler[34] found that plant mitoses also could be blocked, using a saturated aqueous podophyllin. Within the meristem of *Allium cepa*, prometaphases accumulated in the absence of a spindle to complete the division. The chromosomes shortened slightly, but remained attached at the centromere. The picture is like that of a colchicine block, substantiating the similarity reported by King and Maurice Sullivan.

Father T. D. Sullivan[37] found a saturated solution of podophyllin useful for blocking mitoses in petunia leaves. With the addition of 0.05 per cent chloral hydrate, further shortening of the chromosomes could be obtained.

In the meantime, successes with condyloma therapy inspired intensive work with malignant tumors in at least three separate laboratories. At the National Cancer Institute, Dr. Shear's chemotherapy group obtained striking pictures of blocked mitoses peppering whole areas of tumor.[27] The subsequent necrosis eliminated most of the tumor, but viable cells usually remained. Karyokinesis in normal mouse skin and intestine was also blocked by podophyllin.[27] In the chick, the cerebellum was damaged.[28]

Dr. Morris Belkin,[4, 5] at the Medical College of South Carolina, was able

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to reduce the growth of sarcoma 180 to $\frac{1}{7}$ that of the controls and transplantable mammary carcinoma to $\frac{2}{3}$ of control size with injections of podophyllin. Four injections of 20 mg./K. were given every three to four days. Mitosis was arrested at metaphase, presumably leading to the abundant pycnosis and karyorrhexis and the decrease in mitosis found in sections. There was extensive necrosis in both tumors.

At the Sloan-Kettering Institute, podophyllin was already scheduled for investigation as a mitotic poison when a laboratory accident raised our hopes. In one set of tissue culture tubes routinely prepared for the *in vitro* screening program, all the mouse tumor cells were found dead, alongside perfectly healthy control fibroblasts and epithelium. In so far as we could determine, the serum had come from a placenta shed by a mother previously treated with podophyllin. Tissue culture experiments with mouse sarcoma L946 and lung tumor MA387 gave a consistently greater damage to the malignant cells than to normal skin epithelium and fibroblasts.[30, 31] Although it was possible, in the range of 0.6–5.0 mg./L., to kill selectively the malignant cells with a 24-hour exposure, the results were never as dramatic as the wholesale sudden killing of all the tumor explants by the suspected serum. Exhaustive attempts by Dr. Ormsbee to obtain a comparable serum from rats and rabbits injected or poulticed with podophyllin, or from human patients who had had applications of 25 per cent podophyllin in oil, were without success.

A few preliminary observations of damage to mouse tumors MA387 and L946 *in vivo* have been published.[31] Unpublished results from the chemotherapy division at the Sloan-Kettering Institute are included in the discussion.

To obtain quantitative cytological information on the effects of podophyllin on cell division, we turned to marine eggs. Brief notes on *Asterias*[10, 11] and *Arbacia*[12] have appeared previously.

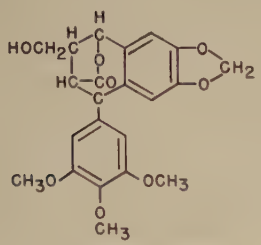
Materials and Methods

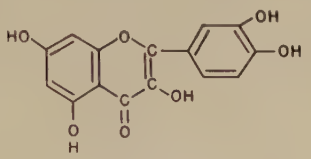
The starfish, *Asterias forbesii*, the sand dollar, *Echinarachnius parma*, and the sea urchin, *Arbacia punctulata*, were used at Woods Hole. At Bermuda, the sea urchins, *Tripneustes esculentus* and *Lytechinus variegatus*, and the sea slug, *Chromodoris*, were used.

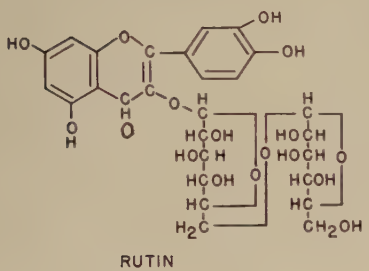
Two known components of podophyllin, podophyllotoxin and quercetin, as well as rutin, a derivative of quercetin, and two derivatives of podophyllotoxin, picropodophyllin and podophyllic acid,[39] were tested. The formulae are shown in FIGURE 1. Also, depodophyllotoxinized resin, *i.e.*, podophyllin from which the podophyllotoxin had been removed, was tested, as well as the chloroform-soluble and chloroform-insoluble fractions of a residue from crude podophyllotoxin, following extraction of the podophyllotoxin. The chloroform-insoluble fraction is equivalent to crude quercetin. The scheme of fractionation is that of Dr. W. G. Bywater, who contributed the podophyllotoxin, picropodophyllin, and fractions of the resin. The pure quercetin used in these experiments was a commercial preparation not extracted from *Podophyllum*. Some data on colchicine are included for comparison.

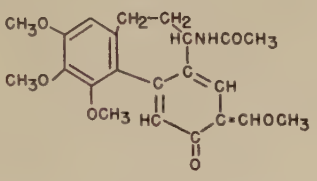
The concentrations tested ranged from 0.0001 to 1.0 mg./L. for podophyllin and podophyllotoxin and its derivatives. The less active quercetin and rutin were used at 0.1 to 100 mg./L. (sat.).

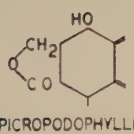
Exposure was begun during the nuclear vesicle stage and maturation divisions in fertilized and unfertilized *Asterias* eggs, as well as before and after fertilization of mature eggs. Sea urchin eggs were exposed before and after fertilization. *Chromodoris* eggs are already fertilized when laid, so no tests could be made on unfertilized eggs. For purposes of comparison between compounds and between species, most exposures were begun 9 to 12











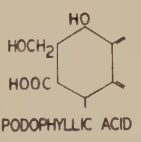


FIGURE 1. The structures of podophyllotoxin,[7, 33] picropodophyllin,[7, 33] podophyllic acid,[6] quercetin,[2, 12] rutin,[42] and colchicine.

minutes after fertilization. Most treatments were continuous, development being followed for one to three days. Shorter exposures for analyzing sensitive periods and recovery ran from five minutes to 12 hours. All observations were made on living eggs, except where otherwise indicated.

Results

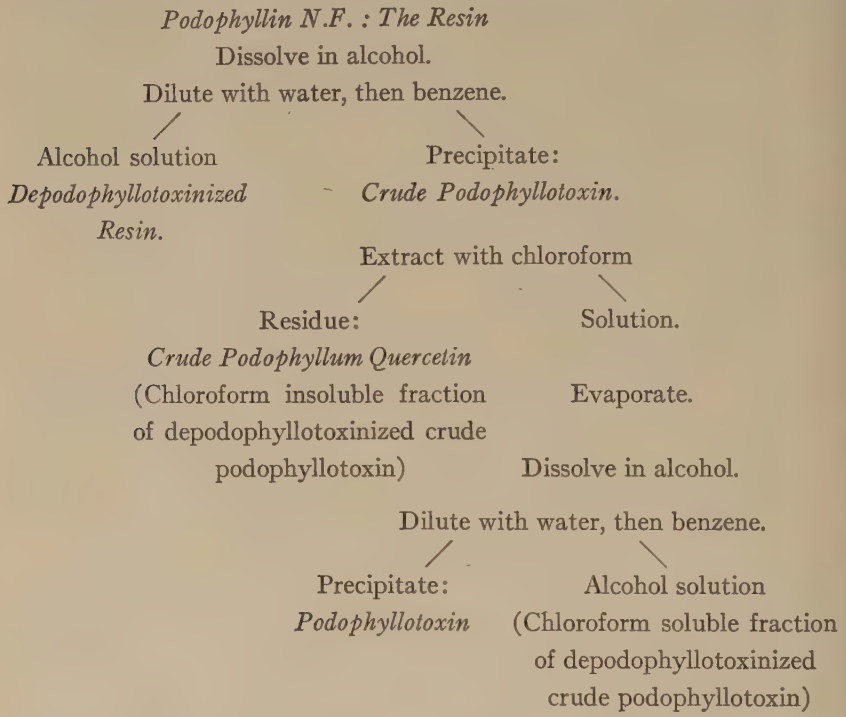
The observations have been grouped according to the species of the experimental animal. This has proved to be the most direct, and it is hoped the clearest, approach to analysis, because each egg has distinctive prop-

erties which suit it for the study of one aspect or another of the cytological effects. A summary terminates the observations for each species.

Arbacia

Eggs were obtained by shedding or by removal of the ovaries, strained through gauze, and washed. The temperature was maintained by running sea water, which ranged from 22° to 23°C. The first cleavage was between 50 and 60 minutes, the second between 28 and 33 minutes later.

A. *Podophyllin.* All experiments at Woods Hole employed a saturated solution (supernatant from a one per cent suspension) of podophyllin.



Dilutions are calculated from the concentration, *e.g.*, 0.1 ml. of supernatant in 10 ml. of sea water is equivalent to 100 mg. of podophyllin per liter. Components in this solution will differ from the proportions in whole podophyllin according to their solubility in sea water.

1. *Exposure Before Fertilization.* Eggs placed in a concentration of 10 mg./L. for six minutes, and then fertilized in the solution, threw off normal fertilization membranes. One or two pronuclei appeared in these eggs, but there was no further development.

2. *Exposure After Fertilization.* Ten minutes after fertilization, eggs were transferred to podophyllin ranging from 0.1 to 10 mg./L. There was no effect at 0.1 and 0.2 mg./L., and only slight delay of cleavage and irregularity of blastulae at 0.4 and 0.6 mg./L. (experiment 4). In another experiment

(5), 0.5 mg./L. permitted only a fourth of the eggs to develop transient furrows and only 1 per cent to divide completely. At 0.7, 0.8, 0.9, and 1.0 mg./L., cleavage was increasingly delayed and irregular (one-sided and unequal). The eggs died without forming blastulae (experiments 1 and 3). Two milligrams permitted 92 per cent development of furrows, which regressed to 32 per cent true segmentation, while at 4 mg./L. only two per cent developed furrows, none dividing completely. There was no visible attempt at furrowing in concentrations of 6 mg./L. (experiment 2). Karyomeres appeared in the blocked eggs.

Different lots of eggs responded similarly, but at slightly different dosages, 2 mg./L. in experiment 1 and 0.5 mg./L. in experiment 5. Experiment numbers are included to permit the reader to judge the validity of a series and to discount the inevitable discrepancies arising from the use of overlapping doses with eggs of different females.

B. *Podophyllotoxin.* Only fertilized eggs were treated. Exposure was continuous, but began at different times before first cleavage.

1. *Early Exposure.* Eggs were transferred to 0.02 to 0.8 mg./L. at 10 to 12 minutes after fertilization. There was no effect at 0.02 and 0.04 mg./L. From 0.05 to 0.1 mg./L., there was increasing delay of cleavage and abnormality of blastulae, but all eggs divided at least several times. The narrowness of the range of sensitivity of any one stage is illustrated in experiment 5. There are few abnormal blastulae at 0.05 mg./L. and few normal blastulae at 0.06 mg./L. (*cf.* also *Lytechinus* dose-effect). At 0.2 mg./L., 65 per cent of the eggs appeared to divide, but the furrows disappeared from half of these. Only one egg in 200 attempted to cleave when the concentration was raised to 0.4 mg./L., but surface deformations resembling abortive furrows continued to appear for several hours. Typically, the furrow was one-sided at these threshold concentrations. Karyomere formation was the rule in blocked eggs. There was no attempt at segmentation at 0.5 and 0.6 mg./L. The molecular weight of podophyllotoxin is 414, so cleavage was abolished at 0.001 mMolar.

2. *Late Exposure.* Using a concentration of 0.5 mg./L., the susceptibility of different phases of the first mitotic cycle was tested by beginning exposure 14, 40, and 47.5 minutes after fertilization. This was equivalent to 36 minutes (fusion of pronuclei), 16 minutes (late prophase), and 12.5 minutes (prometaphase) before 50 per cent cleavage of the respective controls. Cleavage was 0, 23, and 100 per cent respectively, sharply delimiting the end of the sensitive phase at the beginning of metaphase.

C. *Quercetin.* This flavone exerted an effect only near saturation, and the effect varied according to the means of solubilization.

1. *Suspension Directly in Sea Water.* By direct solution, one cannot get 25 mg. to dissolve entirely in one liter. In an attempt to reach complete saturation, a suspension of 100 mg./L. was used. Added to eggs at the "streak" stage, 35 minutes after fertilization (25 minutes before control 50 per cent cleavage), this occasioned an hour delay in the first cleavage, telescoping it into the second cleavage. Normal blastulae eventually formed, but few were motile by 12 hours, whereas motility normally begins about seven hours after fertilization.

2. *Preliminary Solution in Ethanol.* Using 0.4 per cent quercetin in alcohol, one could prepare solutions up to 20 mg./L. Opalescence appeared at 40 mg./L.

At 40 mg./L. (0.125 mMolar), cleavage was blocked. The spindle area remained diffuse in some eggs and, in others, gave way to a well-defined nucleus. The first segmentation was delayed 86 minutes at 20 mg./L. and 31 minutes at 10 mg./L. At these concentrations, cleavage was irregular and asynchronous, but normal blastulae (immobile in 20 mg./L.) finally appeared. Delay decreased with decrease in concentration down to 0.2 mg./L. (0.00067 mMolar), where some lots of eggs gave a few minutes delay and others were unaffected.

To reach a dose of 40 mg./L., it was necessary to introduce one per cent (by volume) ethanol. Simultaneous tests with alcohol of this concentration altered no phase of development. There may have been a slight potentiation of the quercetin by alcohol, as compared with alkali-dissolved quercetin.

3. *Preliminary Solution in Alkali.* A one per cent solution in 0.1 N NaOH was first prepared, quickly neutralized with 0.1 N HCl, and immediately added to sea water in the experimental dishes.

Quercetin so prepared required 200 mg./L. to block all development. At half this, the first cleavage was delayed four hours (400 per cent increment), but a few eggs succeeded in becoming blastulae. Delay of early segmentation decreased with weaker solutions, reaching 18 minutes delay of first cleavage and 37 minutes of second cleavage (29 per cent and 42 per cent increment at 8 mg./L. (0.0265 mM.), the lowest concentration tested.

If the alkaline solution was stored in the refrigerator, it became progressively darker. A test at 24 hours, neutralizing just before use, showed it retained all its potency, 50 mg./L. occasioning a two-hour delay at the first cleavage. After 23 days, however, 50 mg./L. delayed division a debatable few minutes. Nevertheless, it still inhibited ciliary motility down to 10 mg./L., so we have one property abolished by alkali and the other unaffected, suggesting that motility is sensitive to a different part of the molecule or to an alkaline degradation product of quercetin.

D. *Rutin.* Quercetin linked to rutinose (a rhamno-glucoside) is rutin. Only a saturated solution (500 mg./L., 0.775 mM.*) elicited a response, delaying the first and second cleavages about five minutes. The blastulae were normal in structure and activity at 11 hours.

E. *Colchicine.* Colchicine is not a component of podophyllin, but its outstanding value as a mitotic poison, and some resemblance to the podophyllin molecule, justify its inclusion here for comparison. Its effects on the *Arbacia* egg have already been adequately studied.[3, 29, 41] Only a few experiments were needed to establish that the lower threshold determined by the methods used in this series was in close agreement with that found by other investigators. At 10 mg./L. (0.025 mM.), colchicine delayed cleavage a few minutes.

F. Arbacia: *Comparisons.* Podophyllin, podophyllotoxin, quercetin, and colchicine are compared graphically in FIGURE 2. While podophyllotoxin

* Molecular weight 646 (including 2 H_2O). Incorrectly printed in previous abstract.[12]

was ten times more effective than podophyllin, the cytological picture was identical to that seen in eggs poisoned with the cruder mixture. Within a narrow dose range, slowed one-sided, irregular cleavages quickly give way to complete abolition of cleavage before any considerable retardation was attained. Nearly completed furrows regressed at the threshold dose, or with late exposure. In podophyllotoxin, at concentrations which inhibited segmentation, surface deformations resembling abortive furrows continued to appear for several hours. In blocked eggs, karyomeres appeared in place of nuclei. A concentration of podophyllin, double that which halted seg-

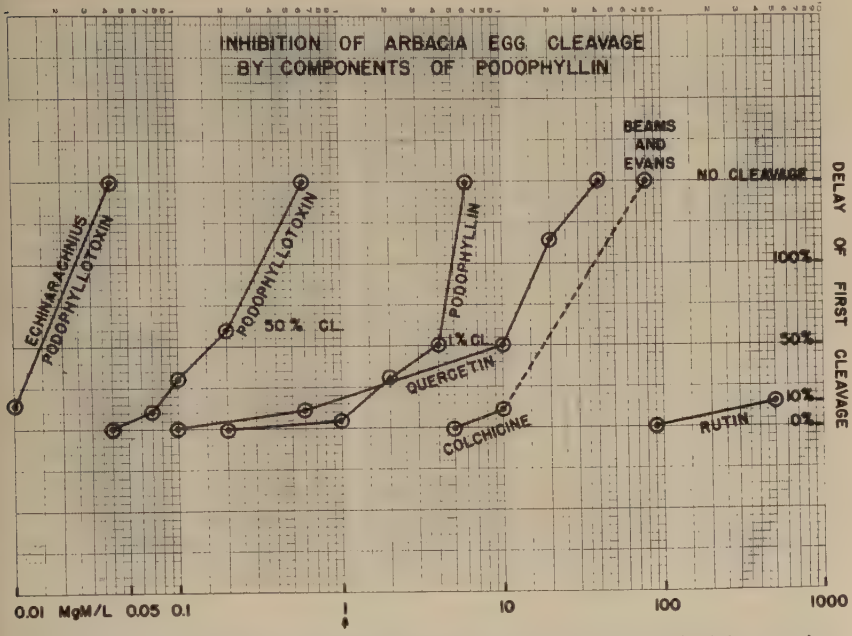


FIGURE 2. Blocking and slowing are arbitrarily included on one scale. Cleavage delay is in terms of per cent increment over control 50 per cent cleavage time. Colchicine has only 1/100 the potency of podophyllotoxin. Quercetin also is weaker than the toxin and podophyllin and differs in being able to retard cleavage several hours without blocking it altogether.

mentation, permitted normal lifting of the fertilization membrane and resolution of a male pronucleus.

Colchicine shows no qualitative difference from podophyllin in its disruption of *Arbacia* development. Persistent chromosome cycles,[29] spindle destruction, karyomere formation, one-sided furrows, and an insensitive period beginning 10 to 14 minutes before cleavage[3] were observed. Prefertilization treatment also produces the same picture with both chemicals (although colchicined eggs were washed). Fertilization membranes form, and pronuclei appear but are slow in fusing.[41] With either, eggs cannot be slowed more than 50 per cent without stopping subsequent development.

Quercetin had some influence at concentrations in the active range of podophyllotoxin but required a much higher dose to block cleavage. This,

spread over a wide range of concentrations, coupled with a persistence of development, even in eggs delayed several hours, typified the quercetin effect. Eggs which cleaved late and even irregularly, formed blastulae which were morphologically normal, lacking only the ability to move. It was necessary to resort to preliminary solution in alcohol or alkali to obtain a concentration high enough to block cleavage. Continued exposure of quercetin to alkali lowered its effectivness against cleavage, but not its ability to immobilize blastulae.

Echinarachnius

The eggs of the sand dollar are not as abundant as *Arbacia* eggs but are more transparent, permitting observation of considerable detail within the living egg. The running sea water used to maintain the temperature ranged from 23° to 24°C. The first cleavage developed at 70 minutes and the second at 110 minutes after fertilization. Treatment was continuous, beginning 10 to 11 minutes after fertilization.

A. *Podophyllin.* Concentrations are calculated as dilutions of a one per cent suspension, taking the supernatant as equivalent to 10 g./L. At 1 mg./L., cleavage was blocked. Within the egg, a hyaline area, resembling an abortive achromatic figure, formed and then faded, leaving one or two karyomeres (or pronuclei). Four mg./L. prevented the appearance of any such configuration. At 0.6 mg./L., the first division was delayed about ten minutes. Cleavage reached an apparent 50 per cent, which regressed to 30 per cent in which the two blastomeres became actually separate. The second cleavage was delayed 40 minutes, and only a minority attained an irregular third or fourth segmentation. In these eggs, the asters, but not the spindle, became indistinct even before cleavage began, and disappeared completely (100 × magnification was employed) between the first and second divisions, although the asters were clearly visible in segmenting control eggs.

With decreasing concentrations, all these manifestations decreased until, at 0.1 mg./L., retardation of cleavages amounted to only a few minutes, asters were only slightly dimmed, and most of the eggs formed blastulae.

B. *Podophyllotoxin.* 1. *Exposure 10 to 11 Minutes After Fertilization.* Eggs made no attempt to cleave when left in a concentration of 0.04 mg./L. (10^{-4}mM.). A vague, less opaque area appeared in the center of the egg and then faded, leaving 1 to 4 karyomeres. In the first experiment, half this dose sufficed to suppress cleavage, permitting only two per cent of the cells to form temporary furrows. In these eggs, the achromatic figure faded into a vague hyaline area about three hours after fertilization. Under phase contrast illumination, this area could be seen to contain several short crooked strands, which may have been remnants of the spindle or possibly clumps of hydrated chromosomes. The hyaline area eventually gave place to 1 to 4 karyomeres. In experiment 2, 0.02 mg./L. merely delayed the first two cleavages a few minutes. Only a fourth of the eggs were able to consummate cleavage in the first segmentation cycle, but most eggs were able to reach the four-cell stage by simultaneous double cleavage at the second cycle. In experiment 1, at 0.01 mg./L., the first cleavage was de-

layed 15 minutes and telescoped into the second cleavage. Eggs were blocked in four- to eight-cell configurations. The eggs of experiment 2 were delayed only a few minutes at 0.01 mg./L. and 19 per cent became blastulae, although abnormal. Significantly, no asters were visible in either experiment at the 0.01 to 0.02 mg./L. level, even in dividing eggs, with ordinary or phase-contrast illumination, although control eggs showed distinct asters. The spindle remained, becoming oval in blocked eggs.

2. *Late Exposure.* When exposure was begun 28 minutes before control 50 per cent cleavage, division was delayed and only 52 per cent formed furrows. These regressed to eight per cent real cleavages and there was no further development. Treatment beginning eight minutes before control 50 per cent cleavage allowed 90 per cent to divide without delay, and later eight per cent three-cell forms appeared.

C. *Quercetin.* Although eggs in 20 mg. L. underwent a peculiar type of surface fragmentation, there was no visible sign of anything that could be called true cleavage. A little before the time for first cleavage, the eggs developed a clear central area which subsequently faded. The surface fragmentation and the same interior zone appeared in eggs remaining in solutions of 10 mg./L., but spindles and asters took form after three hours and divided the egg successfully. Some cells had multiple figures and there appeared to have been simultaneous cleavage into many cells. These eggs produced a few abnormal blastulae.

D. *Echinarachnius: Comparisons.* Eggs were blocked at 1 mg./L. podophyllin or 0.04 to 0.02 mg./L. of podophyllotoxin and were slowed at 0.6 mg./L. of podophyllin or 0.02 to 0.01 of podophyllotoxin. Although eggs from each female showed a different sensitivity, there was a consistent, steep dose-effect relationship among the eggs from a single urchin.

No aster appeared in the heavily dosed dogs, while the spindle rounded up and faded, leaving a few karyomeres. In delayed eggs, the aster disappeared before cleavage began, but the spindle persisted. Treated eggs even continued to divide in the absence of visible asters, a fact of value in interpreting configurations to be described in the *Asterias* egg. Observations on the living egg were not adequate to permit the statement that the asters were completely eliminated, although this very point is well worth detailed investigation.

The insensitive period was obscured by individual variation in cleavage time, but set in between 28 and eight minutes before 50 per cent control cleavage. Every phase of development appeared to be slowed by quercetin, but intermediate doses permitted slow assembling of all the components and activities required for division. With these few experiments, there was no qualitative difference in the response of sand-dollar eggs from those of the sea urchin. It is interesting that these eggs proved to be ten times as sensitive to all three poisons (FIGURE 2 and TABLE 4).

Asterias

The starfish egg is shed in the germinal vesicle stage, affording an opportunity to compare meiosis with mitosis and to analyze effects on a nuclear cycle free from the complications of fertilization.

Ovaries were removed to sea water. Eggs were washed and transferred to test solutions in various stages of maturation and cleavage. As the season advanced, the temperature of the sea water rose from 14° to 20°C. The first cleavage came 155 to 210 minutes after the eggs were shed, in eggs fertilized before maturation was complete. The second cleavage was about 30 minutes later.

A. *Podophyllin.* Dilutions are calculated from the supernatant of a one per cent suspension, which is considered equivalent to 10 g./L.

1. *Cleavage.* (a) *Early Exposure.* First, to compare effects on *Asterias* with the species previously described, we can consider the eggs which have

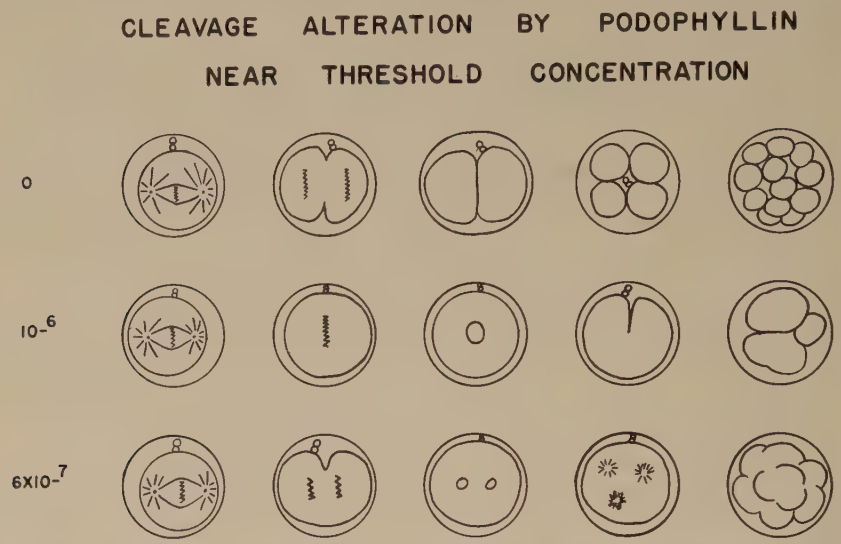


FIGURE 3. At threshold concentrations, the first few cleavages in *Asterias* are by-passed, but irregular multiple cleavages appear one to two hours later. The structures apparently effecting this cleavage are irregular mitotic figures. They are shown as small asters in the diagram, but actually lack astral rays, as seen in the living or sectioned egg. At 0.6 mg./L., a few eggs form an abortive furrow which regresses, leaving two chromosome groups, the chromosomes later becoming irregularly distributed in the multiple achromatic figures. At 1 mg./L., there is no furrow at the time of first cleavage, but later a succession of furrows, or a few simultaneous furrows, divide the egg.

been fertilized and then exposed to podophyllin before or after the second maturation division. This allowed about two hours exposure before the first cleavage was due. There was no effect with a concentration of 0.1 mg./L. Cleavage was slightly delayed and one-sided at 0.2 mg./L., while at 0.4 mg./L. only one per cent completed a normal first cleavage. At this threshold level, there appeared a phenomenon not observed in the sea urchin or sand dollar. At 0.4, 0.6, 0.8, and 1.0 mg./L., the furrows regressed, but then, some time later (90 minutes at 0.6 mg./L.), the eggs underwent multiple cleavage, sometimes into more blastomeres than were in the controls (PLATE 1, 5 and 6). The blastomeres were often multinucleate. This multiple segmentation was preceded by a proliferation of hyaline bodies within the undivided egg (PLATE 1, 1). The process is diagrammed in FIGURE 3, where the hyaline bodies are pictured as diminutive asters. At first, it seemed

entirely probable that these bodies were asters, in view of the frequency with which multiple cleavage is associated with multiple asters in the literature. In the compressed living egg, however, observed at a magnification of 440 ×, no astral rays could be seen. Sections revealed that there were indeed asters of a scraggly appearance. While single asters were seen, others were as often associated with normal or multipolar mitotic figures, so one must suspect that isolated asters were merely the result of sectioning. It was possible, on the other hand, to find mitotic figures completely lacking asters (PLATE 1, 2). The commonest configuration found in slides were small, irregular blotches of basophilic material containing erratically distributed chromatin (PLATE 1, 3 and 4). The bodies seen in the living egg then were not asters alone and did not seem to represent cytasters but were more probably reduced and bizarre figures or, rarely, nuclei and karyomeres. The sections were not all that could be desired, having been taken through a routine formaldehyde fixation and Harris hemalum stain, but it should be emphasized that, in the controls, astral detail was readily visible.

Mitotic figures, nuclei, and naked chromatin were often associated with furrows, but rarely did an aster take a position that could be considered functional with reference to the furrow. So seldom was it possible to detect any chromatin or achromatic material in the small blastomeres formed by multiple budding, that we must question whether these may not be merogons formed by pathological fragmentation, rather than by means of true cleavage furrows.

Sections stained in Harris hemalum and eosin revealed additional basophilic areas which suggested spheres of spindle material containing no visible chromatin, if one were to guess at their nature.

In this range of dosages, normal blastulae were still formed, in decreasing percentages, up to 0.8 mg./L. At 1 mg./L., only ciliated fragments were found.

There were only shallow furrows after two to three hours in 3 and 6 mg./L. Those in 3 mg./L. eventually produced distorted morulae, but by 12 hours the 6 mg./L. series merely bulged irregularly, as if attempting multiple cleavage. At higher doses, furrows did not appear until about 12 hours (20 to 100 mg./L.) and, finally, at 200 mg./L., no activity could be detected. The dose-effect relationships for cleavage and blastulation are graphed in FIGURE 10.

Washing the eggs after two hours exposure permitted incomplete furrowing at 12 hours, even after doses of 2000 mg./L., but did not permit resumption of normal cleavage in the range of 20 to 2000 mg./L.

(b) *Exposure Just Before Cleavage.* When exposure was begun during prophase, the threshold for cleavage inhibition was the same as for earlier treatment. Eggs, placed in 0.8 mg./L. 20 minutes before cleavage, developed 25 per cent furrows by the time the controls had completed cleavage. The furrows then regressed, but nuclear division persisted, giving tetranucleate eggs, with the nuclei close together. At 0.6 and 0.4 mg./L., more eggs developed furrows, and more divided, but again tetranucleate eggs appeared, this time with nuclei well separated. There was a detectable delay in a concentration of 0.2 mg./L., but no abnormality.

To study the susceptibility of different mitotic phases, one test with 100 mg./L. was begun at late prophase. None of the eggs divided, but in some of them, later, two groups of karyomeres showed that these eggs had reached anaphase before being blocked. Another approach was to begin exposure when the furrow had already appeared in 16 to 44 per cent, but no eggs had divided. Samples were fixed at the time of exposure and two hours later. It was found that eggs in any stage of elongation (anaphase), and some which had not begun to elongate (presumably early anaphase), had completed cleavage. In a few of the undivided eggs, there were two groups of karyomeres, revealing that some metaphases had progressed to

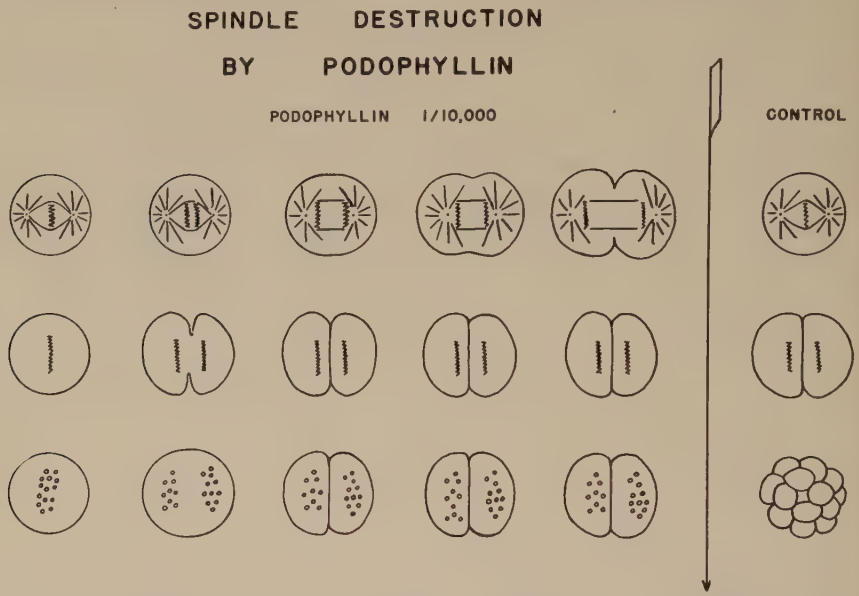


FIGURE 4. *Asterias* eggs in metaphase (27 per cent) and anaphase (73 per cent) were placed in 100 mg./L podophyllin. In the few minutes that podophyllin required to take effect, cleavage progressed to the stages shown in the top row. Destruction of the spindle stopped or reversed cleavage in the metaphase and early anaphase (20 per cent one-cell), but permitted middle and late anaphases to divide (80 per cent two-cell).

anaphase before being stopped. The remainder had been stopped at metaphase (one group of karyomeres). The results are presented diagrammatically in FIGURE 4.

2. *Meiosis.* (a) *Exposure During Second Maturation Division.* Eggs placed in 100 mg./L. podophyllin during the second meiotic anaphase sometimes formed a separate polar body, but they never formed a female pronucleus. In its place, beneath the polar body, there were karyomeres averaging ten in eggs with two polar bodies (PLATE 3, 17) and reaching a maximum count of 16, the haploid number. In eggs with only one polar body, about 20 karyomeres could usually be found and the best count was 30, less than 2n. These karyomeres disappeared in eggs that were then fertilized and reappeared in larger numbers, but seldom doubled.

A cycle resembling parthenogenesis could also cause the karyomeres

to disappear. This was best demonstrated in unfertilized eggs which had matured and formed a pronucleus before they were treated. In some series, a majority of the eggs lost their pronuclei, and later a cluster of karyomeres appeared. Usually, this was accompanied by the lifting of a "fertilization" membrane. Pronuclei inactive as long as 3.5 hours would, under the aegis of slight mechanical stimulation, dissolve and form karyomeres while still in podophyllin of 100 mg./L.

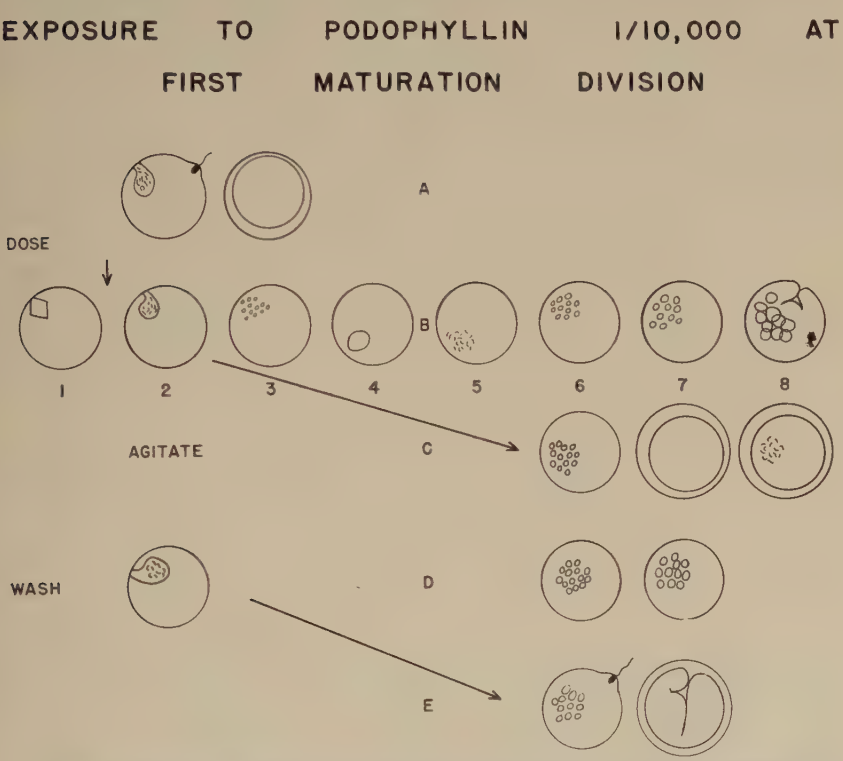


FIGURE 5. Eggs were exposed when the first polar spindle had appeared. Eggs continously exposed underwent two chromosome cycles (B2 and 5) with intervening karyomere and spindle (?) formation. There were abortive furrows at 24 hours (B8). Fertilization just after exposure hastened the first cycle (A). Spontaneous parthenogenesis precipitated a third cycle (C). Washing and fertilizing also started a third chromosome cycle with successful but irregular cleavage (E).

(b) *Exposure During the First Maturation Division.* When one poisoned an egg containing a first meiotic spindle, the spindle immediately rounded into a sack-like structure within which one could sometimes see small refractile bodies (FIGURE 5, B-2). These bodies were presumably chromosomes, but no special stains have as yet been used at this stage to determine the presence of chromatin. The rounded spindle dissolved, leaving the egg empty within 12 minutes. By 25 minutes, tiny karyomeres had made their appearance, and these in turn were replaced by an oval hyaline body at about 1.5 hours. In sections, this oval body was without visible structure and stained so densely that chromatin, if present, could not be distinguished (PLATE 2, 7).

The picture was compatible with one of dense but unorganized spindle material. It could, however, have been an irregular nucleus taking a heavy hematoxylin stain, just as the karyomeres did later (PLATE 2, 8). An hour later, this body also dissolved, leaving tiny refractile dots which slowly grew into karyomeres. These were always 20 to 30 in number, about double the counts in eggs poisoned after the second maturation division. The karyomeres continued to grow, but, in some eggs, still a third chromosomal cycle was imposed, coincident with the lifting of a membrane. It is suspected that this represents a parthenogenetic cycle activated by agitation or CO_2 accumulation (FIGURE 5 row C).).

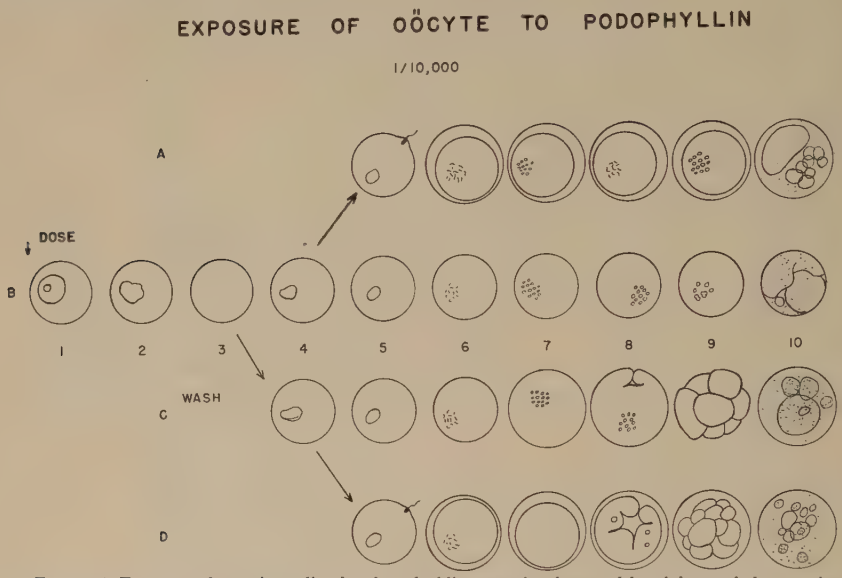


FIGURE 6. Exposure of eggs immediately after shedding permitted normal breakdown of the germinal vesicle, and one or two chromosome cycles *via* an intermediate "spindle" (B5). Fertilization (A), removal from podophyllin (C), or both (D) improved the prospects of cleavage and hastened the second chromosome cycle. As the karyomeres grew, they sometimes fused (B9). The hypertrophied karyomeres remained intact in cytolysed eggs (A10). The egg in D6 retaining a tetraploid chromosome set from both maturations, and gaining the sperm complement as well, should be pentaploid.

If one fertilized the eggs just after the spindle had begun to round up, an acceleration of the first chromosome cycle was announced by the complete disappearance of chromosomes and spindle (FIGURE 5, row A). Washing out the podophyllin after 14 hours exposure had little effect on the sequence of events (FIGURE 5, row D), but fertilizing washed eggs incited karyomere solution and brought about an irregular cleavage (FIGURE 5, row E) which never resulted in embryos. Shallow one-sided furrows appeared much later in unfertilized eggs under continuous exposure (FIGURE 5, B-8).

(c) *Exposure of the Oöcyte.* When freshly shed eggs were placed in strong (100 mg./L.) podophyllin, breakdown of the germinal vesicle proceeded unaltered in speed or pattern. These events are diagrammed in FIGURE 6. A highly refractile body appeared, identical in living and in sectioned eggs

to that which evolved when treatment was begun later in the cycle (FIGURE 5, B-4). Whether this was equivalent to the second or first meiotic spindle (assuming these bodies to be abortive spindles) could not be decided. Closer timing might have revealed two successive "spindles." The chromosome cycle that followed was identical to that found with exposure during the first maturation division: refractile bodies giving rise to karyomeres (PLATE 3, 13, 14). These grew and, in one experiment, fused (PLATE 3, 15), eventually forming a single nucleus with a large nucleolus (PLATE 3, 16). Those that continued to grow formed dense, homogeneously basophilic nuclei (PLATE 2, 8). A second chromosome cycle ensued in fertilized eggs (FIGURE 6, A-8, D-7), and in one experiment a spontaneous second cycle at 10 to 11 hours brought the number of karyomeres to 50 to 60. By 12 hours, the unfertilized, continuously exposed eggs had fragmented in a way that at least resembled erratic segmentation. Better and earlier cleavage was obtained by transfer to unpoisoned sea water (PLATE 2, 9), by fertilizing (PLATE 2, 10, 11; FIGURE 6, C-8, 9), or by both (PLATE 2, 12, FIGURE 6, D-8, 9). Eggs observed during this late division were seen to be in a constant state of flux, with old furrows fading and new ones pinching in. All eggs cytolysed without getting beyond an irregular morula stage. This served to reveal the structural integrity of the karyomeres, which floated intact in the cytoplasmic debris (FIGURE 6, A-10).

"Parthenogenetic" membranes and nuclear breakdown appeared also in eggs of this series, when under continuous exposure. An attempt to accentuate this process with 2.5 M NaCl, or by agitation, did not alter the percentage of membranes.

(d) *Exposure After Fertilization.* The previous experiments are primarily concerned with the behavior of the female chromosomes. To compare the action of podophyllin on the sperm nucleus, eggs were fertilized during the second maturation division, then placed in podophyllin (100 mg./L.) 20 to 40 minutes later. In some eggs, a small pronucleus appeared, at some distance from the chromosomes released by the maturation spindle under the polar bodies (FIGURE 7, A-2). In the same relative positions there developed two groups of 6 to 10 karyomeres. At least one chromosome cycle was detected, involving disappearance and reappearance of the karyomeres (A-4). As with other treatments, the karyomeres increased in size. The eggs developed furrows at 12 hours, and if washed free of podophyllin, divided several times (A-6 and B-6).

B. *Podophyllotoxin.* Unfertilized eggs were placed in 10, 1.0, 0.1, and 0.01 mg./L. of podophyllotoxin during the second maturation division. The changes were not followed closely, but, at four hours, some eggs had 10 to 20 karyomeres, while an occasional cell showed small refractile bodies, suggesting that a nuclear cycle was in progress. The karyomeres enlarged and fused. Only in 1 mg./L. at 10 hours was there any furrow formation.

C. *Quercetin.* Concentrations of 10, 1.0, 0.1, 0.01 mg./L. of quercetin were added to the eggs just after completion of the first polar body. The top concentration destroyed the spindle, permitting development of karyo-

meres in eggs with one polar body. These karyomeres were larger at 6.5 hours. Eggs were able to complete only one maturation division also in 1 mg./L., but the chromosomes formed a nucleus immediately beneath the polar body. At 0.1 mg./L., the egg completed both divisions, but the pronucleus remained eccentric, often immediately beneath the polar bodies. Maturation was entirely normal at the lowest concentration: 0.01 mg./L.

D. *Colchicine.* For a final comparison, tests were run with colchicine, encompassing concentrations of 100, 50, 10, 5, and 1 mg./L., beginning the treatment during the second maturation division. After two hours, the cleavage percentages read: 0 mg./L., 95 per cent; 1 mg./L., 83 per cent; 5 mg./L., 1 per cent (unequal). In 10 and 50 mg./L., there were one to three

FIGURE 7. The eggs were fertilized when the second maturation spindle was visible and transferred to 100 mg./L. podophyllin 20 to 40 minutes later. Formation of a female pronucleus was prevented (A2) and the chromosomes hydrated to form approximately the haploid complement of karyomeres (A3). The sperm head became visible as a temporary pronucleus (A2), which then dissolved and reappeared as a second haploid set of karyomeres (A3). Both female and male karyomeres then dissolved (A4), reappeared, and hypertrophied. There was furrowing at 12 hours (A6). Returning the eggs to untreated sea water after two hours exposure (B3) permitted more extensive subdivision of the egg, with unequal apportioning of the karyomeres (B6).

irregular hyaline areas resembling distorted mitotic figures. The lowest dose formed normal gastrulae, but at 5 mg./L. there were only blastulae with a solid interior, resembling centrolecithal eggs. The higher doses permitted no cleavage or blastogenesis.

Exposure to 100 mg./L. at the germinal vesicle stage, or during the first maturation division, prevented subsequent meiosis and precipitated the chromosomes into the cytoplasm, where they slowly grew into gigantic karyomeres. There was at least one nuclear cycle in some of the eggs subsequent to exposure. By 14 hours, there was abnormal cleavage or fragmentation resembling cleavage.

E. *Asterias: Comparisons.* Continuous exposure of the fertilized egg to podophyllin, beginning two hours before cleavage, had no effect at a level of 0.1 mg./L. Segmentation was delayed and one-sided at 0.2 mg./L. At 0.4 to 0.8 mg./L., the normal cleavage sequence was supplanted by a period

of furrow formation and regression, followed by proliferation of abnormal mitotic figures and eventually by multiple cleavage which subsequently produced blastulae. We can say, then, that furrow formation is more sensitive than blastula formation, the inverse of these sensitivities in *Arbacia.* From 3 mg./L. up to 100 mg./L. there was still shallow furrowing or bulging over a period of several hours, but no cleavage.

The threshold was the same when exposure was begun after the mitotic figure had appeared. Exposure to higher doses revealed that mitoses caught at metaphase or early anaphase were unable to partition the cytoplasm. Karyomeres appeared in the blocked cells.

Dose-effect comparisons established that effectiveness increased about a hundred times with a doubling of concentration, a ratio comparable to that in *Lytechinus.*

Poisoning the unfertilized eggs with podophyllin before or during the maturation divisions eliminated the meiotic spindles and forced the chromosomes to form individual karyomeres. The karyomeres underwent cycles of dissolution and reformation in response to internal rhythm, or stimulation by fertilization, or by parthenogenetic activation. The karyomeres could grow and fuse, and all visible aspects of fertilization and maturation not dependent upon the achromatic figure continued normal in blocked eggs. The sequence of events depended upon the stage at which exposure to podophyllin began, the most important difference being the appearance of the haploid number (16) of karyomeres in eggs caught at the end of the second maturation, whereas, following earlier exposure, nearly the diploid number could be counted. At 100 mg./L., the sperm head was able to form a pronucleus which subsequently dissolved and was replaced by karyomeres.

Fertilizing the eggs or removing the podophyllin permitted better cleavage, but it was always delayed or abnormal in eggs that had received an effective dose.

The results with podophyllotoxin and colchicine were the same as with podophyllin, in so far as they were studied. With quercetin, it was possible to eliminate the first meiotic spindle and induce karyomere formation at 10 mg./L. *Asterias* was more sensitive to all four substances than was *Arbacia,* and about as sensitive as *Echinarachnius.*

Tripneustes

Considerable difficulty was experienced in fertilizing some lots of eggs early in the season (November, 1947). Where there was good fertilization, slowness of the fertilization reaction still seemed to be reflected in variation in the time of 50 per cent cleavage (82 to 94 minutes at 25°, 95 to 105 minutes with second cleavage 50 to 65 minutes later at 23°C), and in the spread of 10 to 30 minutes between 10 per cent and 90 per cent division.

The eggs of this Bermuda urchin are as transparent as those of *Asterias,* permitting observation of the grosser cytological events. Also, a larger series of podophyllin fractions and derivatives had become available, making possible a wider range of comparisons.

All experiments were run at 23° ± 0.1°, except the first two of podophyllin (0.01 to 3 mg./L.), podophyllotoxin (0.01 to 2 mg./L.), and picropodophyllin (0.05 to 2 mg./L.), which were held at 24.5° ± 0.5°C.

A. *Podophyllin.* A one per cent solution in alcohol served as a stock solution for these experiments, and the first dilution was made with a large amount of sea water. The eggs were thus exposed to more nearly the same proportion of components as occur in dry podophyllin than was possible using a saturated aqueous stock solution. Exposures were begun 10 to 15 minutes after fertilization.

1. *Graduation of Effects.* A scale of cytological criteria was devised in order to compare effects of this series of substances on different species. Three levels of response are detectable in *Tripneustes* eggs. As a rule, these levels reflect the action of doses at successive orders of magnitude. Each response type is subdivided according to severity.

Complete Block (*A*). None of the eggs divide. There may be complete suppression of cytoplasmic activity (a), or eggs may show delayed (6 to 12 hours) furrowing or wrinkling, apparently truly vital activity, but often prefatory to cytolysis and disintegration (b). A few eggs may desultorily attempt cleavage during the first six hours after fertilization (c).

Incomplete Block (*B*). It is typical of some poisons to permit the eggs to initiate cleavage furrows, often nearly synchronously with the controls, but these furrows disappear (a). With some treatments, a few eggs divide once or twice. It has been helpful to set off this type of response arbitrarily at 5 per cent or less cleavage (b).

Slowing (*C*). Where the poison asserts itself by prolonging the time between fertilization and cleavage, we are best able to express the physiological response quantitatively. Delay is measured from 50 per cent control cleavage to 50 per cent experimental cleavage. Where cleavage of the exposed eggs reaches only 10 to 60 per cent, comparison is made between the straightest parts of the cleavage curves, *i.e.*, (a) extreme delay, cleavage time doubled (delay 1.5 hours with *Tripneustes* at 23°C); (b) definite delay 10 to 100 per cent increase in time to 50 per cent cleavage; (c) slight delay, less than 10 per cent. This is an uncertain parameter with material as variable as *Tripneustes* eggs, but it can usually be verified at the second cleavage or blastula stage, *i.e.*, (d′) in a few instances, a drug without effect on the first cleavage completely blocks development by the second or third cleavage. Such a sequence has special implications deserving a separate category, Cd′. Another category is: (d) where cleavage first is not detectably delayed, but second cleavage is slowed. Where this is merely evocation of a slight effect by virtue of a second opportunity to assert itself, it is of interest only in delimiting the lowermost threshold. However, it serves also to reveal compounds of low penetrability (at the plasma or nuclear membrane) or those with delayed action.

Delayed Effect (*D*). When the first two cleavages are not affected, but later stages are delayed, distorted, or killed, the activity is placed in this lowest category.

2. *Variability in Response.* Podophyllin slowed *Tripneustes* cleavage at 0.0005 to 0.004 mg./L. and blocked completely at 0.002 to 3.0 mg./L.

The overlap is largely the result of variability in eggs from different females responding to a drug with a narrow threshold. In TABLE 1, the pattern of dose and effect is spotty, reflecting this variability. Inconsistencies were encountered only between different spawns, not among eggs from a single female.

Nothing new was revealed in the cytology of treated eggs. Karyomeres were formed at the high doses, while irregular multiple division figures and temporary furrows were found in concentrations producing an incomplete block.

TABLE 1
VARIABILITY IN RESPONSE OF *Tripneustes* TO PODOPHYLLIN
(CONCENTRATIONS IN MG./L.)

A: Complete block			*B: Incomplete block*		*C: Retardation*			*No effect*
a	*b*	*c*	*a*	*b*	*a*	*b*	*c*	
	3.0 1.5 0.1							
	0.016	0.04 0.02 0.01						
0.002		0.004		0.008 0.004 0.001	0.002 0.001	0.004	0.002	0.002 0.0016 0.001
					0.0005			0.0008 0.0005 0.0004 0.00025 0.0002 0.0001

B. *Podophyllotoxin.* Exposures were begun 10 to 15 minutes after fertilization. The variability of response is listed in TABLE 2, following the pattern of TABLE 1. The lowest concentration producing an effect in any experiment was 0.0005 mg./L. (10^{-6} mM.).

The cytological effects were the same as those observed with podophyllin, and with podophyllotoxin in other forms. There were large karyomeres, irregular hyaline areas like distorted mitotic figures, and delayed (12 hours), incomplete furrows in 0.001 to 0.05 mg./L.

Eggs placed in 0.06 mg./L. as late as prophase (66 minutes after fertilization) did not divide. They formed only three to six karyomeres, in contrast to the 20 or more obtained with other treatments.

C. *Picropodophyllin.* This isomer of podophyllotoxin blocked cleavage completely at 1 and 4 mg./L. and incompletely at 2 mg./L. (0.005 mM.), the

discrepancy arising from comparison between different lots of eggs. There was a six-hour delay at 1 mg./L., one hour at 0.5 and 0.2 mg./L., and 10 to 20 minutes at 0.1 and 0.2 mg./L. No effect was detected with 0.05 mg./L. All exposures began ten minutes after fertilization.

Karyomeres were the typical end result of blocking cleavage.

Where picropodophyllin and podophyllotoxin were tested on the same lot of eggs, the latter proved 40 to 250 times more effective (TABLE 3).

TABLE 2

VARIABILITY BETWEEN DIFFERENT LOTS OF *Tripneustes* EGGS IN THEIR RESPONSE TO PODOPHYLLOTOXIN (CONCENTRATIONS IN MG./L.)

A: Complete block			*B: Incomplete block*		*C: Retardation*		*D: Delayed effect*	*No effect*
a	*b*	*c*	*a*	*b*	*a*	*b*		
	0.5 0.2 0.1							
	0.04	0.08 0.04 0.02						
				0.01		0.01		0.01
	0.008		0.004					
				0.002 0.001		0.002	0.002	0.002 0.001
					0.0005			0.0005 0.0001

TABLE 3

COMPARISON OF PICROPODOPHYLLIN AND PODOPHYLLOTOXIN IN SIMULTANEOUS RUNS WITH *Tripneustes**

	Complete block	*Incomplete block*	*Retardation*
Picropodophyllin	1.0 mg./L.	2.0 mg./L.	0.2 mg./L.
Podophyllotoxin	0.004	0.05	0.002
Factor	250	40	100

* Despite sensitivity differences in the eggs of different females, podophyllotoxin was always more effective by a factor of 40–250 times.

D. *Podophyllic Acid.* Hydrolysing the lactone ring of picropodophyllin decreased its potency. Beginning 12 to 15 minutes after fertilization, 10 mg./L. were required to block cleavage, and 2 to 5 mg./L. suppressed nearly all division. Retardation of the first cleavage amounted to 25 minutes in 1 mg./L. and four minutes in 0.5 mg./L. (0.001 mM.).

Karyomeres appeared in blocked eggs, and there was surface deformation at ten hours reminiscent of the behavior in all other podophyllins.

E. *Depodophyllotoxinized Resin.* Exposures began 10 to 12 minutes after fertilization. This depodophyllotoxinized resin blocked cleavage at 0.01 to

0.04 mg./L., although the cell surface tardily showed wrinkling and deformation that may have been shallow furrows. The block was incomplete at 0.005 to 0.02 mg./L. In one experiment, the uncleaved cells in 0.02 and 0.04 mg./L. developed large karyomeres in addition to multiple small achromatic figures. Retardation at 0.01 mg./L. was about three hours in one experiment. The mitotic figures persisted as monasters or diasters throughout the period of inactivity. In another experiment, the first cleavage was delayed only 35 minutes, but barely six per cent formed furrows, and these regressed to 0.5 per cent. Much later, the eggs continued their cleavage, but never formed blastulae. There was a few minutes delay of first or second cleavage at 0.005, 0.0025, and 0.001 mg./L.

F. *Fractions of Depodophyllotoxinized Crude Podophyllotoxin. Chloroform-soluble.* Cleavage was stopped at 0.05 and 0.005 mg./L. in one experiment and incompletely blocked at 0.005 mg./L. in another. In the first experiment, there was a slight delay of division at 0.0005 mg./L., but not in the second.

Chloroform-insoluble. This fraction blocked cleavage only at 0.05 mg./L. and was without effect at 0.005 mg./L.

G. *Quercetin.* Exposures were begun 10 to 16 minutes after fertilization. Only at 12.5 mg./L. was it possible to get a retardation of cleavage. There was a seven-minute retardation in one experiment, and a 33-minute retardation in another. Cleavages were irregular, but normal blastulae resulted. At 25 mg./L., cleavage was delayed four hours but eventually produced six per cent solid morulae which were still immobile at 22.5 hours. In delayed eggs, a normal division figure developed slowly, sometimes over a period of hours. There was no karyomere formation or dissolution of the achromatic figure as with the podophyllins. There was no detectable response at 5 mg./L. and lower.

H. *Colchicine.* Using colchicine as a base line for comparison of mitotic effects, we can briefly list the effective concentrations. Exposure was begun ten minutes after fertilization. Cleavage was completely inhibited at 10, 40, and 80 mg./L. Cleavage was incompletely inhibited (one per cent three-cell at 9.5 hours) at 5 mg./L. Cleavage was retarded several hours at 2, 3.6, 5, 7.2, 10, and 20 mg./L., and a few minutes at 1 mg./L. (0.0025 mM.), and second cleavage was delayed three minutes at 1.8 mg./L. No effect was observed at 0.25, 0.45, 0.5, 0.9, 1, and 2 mg./L.

As with the podophyllins, sensitivity varied from sample to sample of eggs. There was karyomere formation in both entire and undivided eggs poisoned with 2 and 5 mg./L. in one experiment.

I. *Tripneustes: Comparisons.* The cytological effects of colchicine and the isomers and derivatives of podophyllotoxin were like those of podophyllin. The spindle was destroyed at high concentrations, leaving karyomeres behind. At threshold levels, temporary furrows appeared and, within the egg, irregular, often multiple, areas like distorted mitotic figures could be seen. There was deformation of the cell surface at 10 to 12 hours. Every phenomenon was not recorded for every substance, but the similarity of the general picture was such as to promise that events would be the same had it been possible to follow a larger number of concentrations in detail. Podo-

phyllotoxin was perhaps slightly more effective than crude podophyllin, while picropodophyllin and podophyllic acid were successively less active.

Depodophyllotoxinized resin resembled both podophyllotoxin (karyomere formation and regression of furrows) and quercetin (persistent achromatic figures and extreme delay of cleavage) in cytological effects, but its potency in some experiments was equal to that of podophyllin.

The chloroform-soluble fraction of the depodophyllotoxinized residue from crude podophyllotoxin was as effective as podophyllotoxin itself, while the chloroform-insoluble fraction was about a tenth as strong. This fraction is mostly quercetin, but it obviously carries a more potent component. Quercetin did not block division even in saturated solutions. It retarded cleavage and immobilized blastulae at 25 mg./L.

Lytechinus

The egg of this Bermuda urchin is one of the most transparent available. It was, therefore, used to clarify the cytological picture of podophyllin effects, and to attempt a few preliminary analyses of the kinetics of podophyllin action. All experiments were at 23.0° ± 0.1°C. At this temperature, 50 per cent first cleavage usually came at about 60 minutes after fertilizing, but varied from 55 to 65 minutes. Regardless of the time of first cleavage, the second followed in 30 minutes.

A. *Podophyllin.* Dilutions were prepared from one per cent in alcohol, the same as for *Tripneustes.*

1. *Exposure 10 to 13 Minutes After Fertilization.* At 0.001 to 0.02 mg./L., cleavage was not delayed and the blastulae were slightly irregular. At 0.04 to 0.08 mg./L., cleavage was delayed or blocked, depending on the egg sample. At 0.1 mg./L., cleavage was blocked (0.00025 mMolar).

Karyomeres were a constant feature of blocked eggs. Growth and redissolving of the karyomeres was observed, but these nuclear cycles were not followed in detail. Late multiple bulging, as if an unsuccessful attempt at segmentation were in progress, was noted at four hours in 0.1 mg./L. Karyomeres were not visible in most of these eggs.

2. *Podophyllin-Sensitive Period of First Cleavage.* Exploratory experiments with other species (*q.v.*) showed that exposure of eggs to a high concentration of podophyllin or podophyllotoxin just before cleavage had no effect on rate or percentage of cleavage. The attempt was made to define the boundary of the sensitive and insensitive phases by using delayed continuous and short exposures.

(a) *Continuous Exposure.* Transferring the eggs to a 0.1 mg./L. concentration of podophyllin at 42, 37, 32, 27, 26, 25, 22, and 20 minutes before 50 per cent control cleavage, blocked division completely. When the mitotic figure had already developed, it faded, leaving two chromosome groups and a few astral rays (or cytoplasmic casts of the rays) but no spindle. The abandoned chromosomes formed nuclei or karyomeres.

A few eggs divided when exposure began 22, 21, 20, 16, and 15 minutes before 50 per cent cleavage, but most of the eggs which started to divide

regressed to a binucleate unicellular condition. This regression of furrows was a striking phenomenon when a population that was to all appearances mostly two-celled suddenly reverted to a few per cent complete cleavage (FIGURE 8, C). Exposure at 11, 10, and 9 minutes before cleavage neither delayed nor blocked the first cleavage, but there was no second cleavage. Even 1 mg./L. at nine minutes caused no delay.

(b) *Short Exposure.* The insensitive period, extending over ten minutes preceding cleavage, could mean merely inadequate penetration within that time interval. Accordingly, eggs were exposed for ten minutes at an earlier part of the cycle, beginning 59 minutes and 38 minutes before cleavage. The eggs were then transferred to a large volume of podophyllin-free sea water, but segmentation was blocked and never resumed, beyond a few delayed irregular divisions which may have been pathological fragmentation.

This was in an early part of the cycle, however, and one has to answer the argument that permeability may have been higher then. To cover this eventuality, eggs were exposed for five minutes, beginning 26, 21, 16, and 11 minutes before 50 per cent cleavage (FIGURE 8). Thus, the latest set was removed from podophyllin when only one per cent of the control eggs had divided, and six minutes before 50 per cent cleavage. The eggs of this last set were not delayed or abnormal at the first cleavage, although the second cleavage was delayed and abnormal (FIGURE 8, DW). Thus, most of the furrows for the first segmentation formed and completed division unaltered, although the eggs already contained enough podophyllin to affect segmentation. Eggs exposed at the same time, but left in podophyllin, divided normally and synchronously with the controls, then did not divide a second time. The preceding exposure, extending from 16 to 11 minutes before cleavage, delayed segmentation and caused many nearly-divided eggs to regress to a one-celled condition. They then successfully reached a two-cell status during the second cleavage cycle, which was even more delayed. The delay is shown in curve CW of FIGURE 8, but regression is not shown in order to keep delay uniformly represented (*cf.* explanation of FIGURE 8 and footnote*). However, regression of cleavage in eggs exposed continuously, beginning at 16 minutes, can be read from curve (C). Delay of first cleavage increased as more time was allowed between exposure and cleavage (AW, BW), but exposures at 26 and 21 minutes (AW and BW) retarded the second cleavage less than exposure at 16 minutes (CW). Embryogenesis was abnormal. All the washed eggs formed at least a few irregular gastrulae.

(c) *Sensitive Phase.* Observation of nuclear dissolution at the end of prophase and correlation of the *Lytechinus* cleavage cycle with Fry's time table for *Arbacia*[17] establishes midmetaphase as the beginning of the insensitive period of the mitotic cycle, 10 to 11 minutes before cleavage. In any

* In evaluating delay of segmentation, one must decide what shall constitute the first cleavage proper. With threshold doses and with delayed exposure, many eggs almost complete cytokinesis, then lose the furrow. At the next cleavage cycle, as the divided eggs are becoming four-celled, the one-celled eggs become two- or four-celled. The delayed transition from one to two cells can only be considered part of the second mitotic cycle. For quantitative study of percentage of cleavage and retardation, only eggs passing from one- to two-cell within the time delimited by the distribution curve of the first division were counted as first cleavage. The same strict interpretation was applied to second cleavage. These concepts are particularly important for interpreting the results of delayed exposure (FIGURE 8) and dose-effect relationships (FIGURE 10).

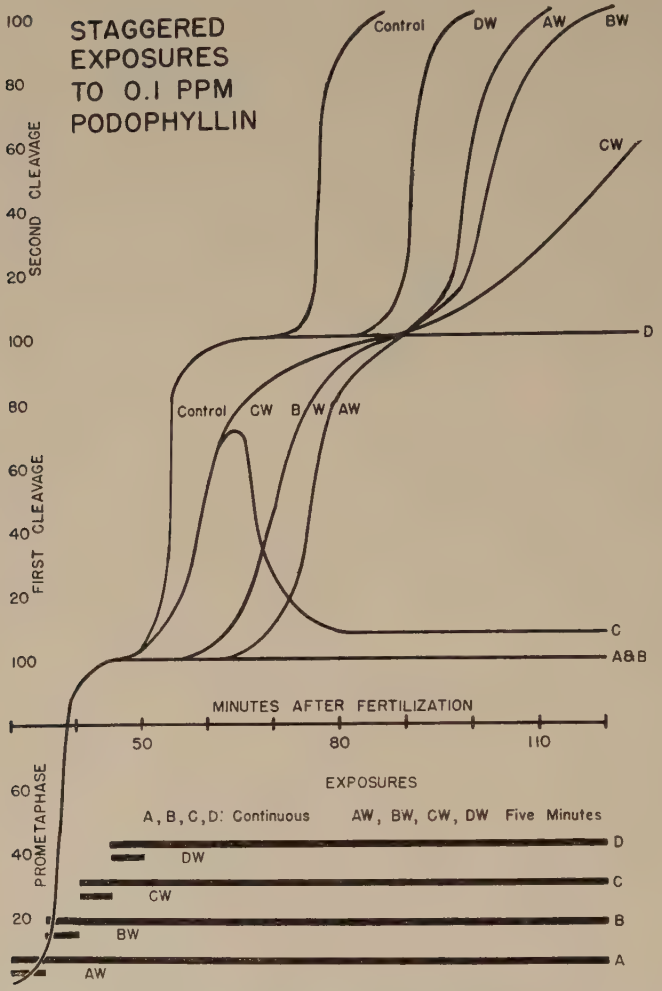


FIGURE 8. This is a schematic representation of an experiment showing blocking of cleavage by continuous exposure and delay of cleavage by five-minute exposures, beginning 30, 35, 40, and 45 minutes after fertilization. The ordinate indicates progress through three stages: prometaphase of the first mitosis, first cleavage, and second cleavage.

There was no cleavage with continuous exposures A and B and no second division in D, while, in C, a majority underwent quasi-division but regressed to eight per cent true cleavage.

To simplify the representation of retardation, 100 denotes whatever final percentage of eggs that divided during the first and second cleavage cycles (*cf.* footnote p. 1465).

In AW, BW, and CW, some eggs did not attempt to divide in the first mitotic cycle, and half of those which appeared to have completed division reverted to single cells. Eventually, all the eggs which had been exposed for five minutes divided successfully. This regression and subsequent division are not included in the curves for the short exposures, which are intended merely to illustrate extent of retardation of the cleavage cycles. CW, BW, and AW were progressively more retarded at the first cleavage, whereas, during the second cleavage, CW was the slowest, extending off the scale of the chart. The critical curve is DW, showing that between 45 and 50 minutes podophyllin had entered the egg in amount sufficient to delay the second cleavage, but it did not affect the first cleavage, which had not begun in most eggs by 50 minutes.

population of cells where the median had reached prometaphase (FIGURE 9, B-1, FIGURE 8, C), the more retarded eggs were blocked (FIGURE 9, A) and the more advanced divided, though sometimes with imperfect polar migra-

tion of the chromosomes (FIGURE 9, C), while the majority almost completed cleavage, then regressed to a binucleate one-celled state.

2. *Prefertilization Exposure.* The rapid sequence of events in an egg with its mitotic cycle already under way prevents detailed observation of induced changes. Particularly, one finds it hard to know whether a spindle or aster under observation is a functioning organelle or a relic that has not yet disappeared into the cytoplasm.

To test both the formation and functioning of the aster, eggs were placed in podophyllin at 0.1 or 0.2 mg./L. at 20 to 34 minutes before fertilization. Activation of the egg by the sperm remained normal, except for the un-

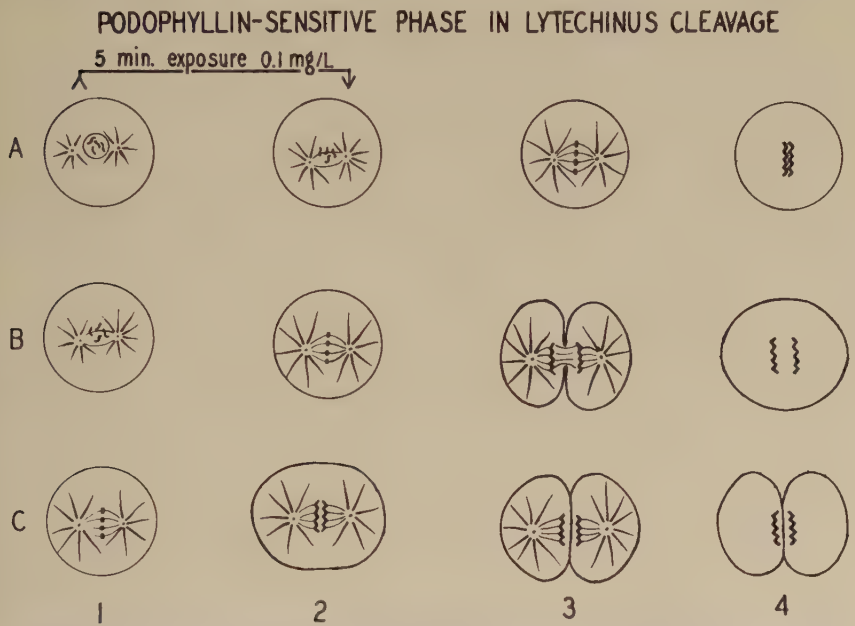


FIGURE 9. This represents a population of cells comparable to that in FIGURE 8-C. Most of the cells are in early metaphase (B) when exposure begins. Such cells proceed to anaphase, where the furrow forms and regresses. More advanced eggs (C) complete cleavage. The slower eggs (A) are blocked completely.

usually large fertilization cone. The sperm formed a pronucleus. Around one of the pronuclei, astral rays, or at least radiating lines, could be traced in the living egg at 30 to 60 minutes after fertilization. The male and female pronuclei remained separate in most eggs, but one received the impression that the pronuclei drifted closer. Fusion of at least some pairs of pronuclei was repeatedly verified. It was not possible, however, to ascertain any drift by statistical comparisons of the distances separating the nuclei in the short time between the appearance of the hydrated sperm head and the dissolution of the pronuclei. Tracing the paths in single eggs[9] would be necessary to establish whether the observed rays were functioning in this way.

3. *Recovery.* It was mentioned in a previous section that eggs exposed to

0.1 mg./L. podophyllin for five to ten minutes during the first cleavage cycle recovered to the extent that some produced abnormal gastrulae.

Exposing eggs before fertilization gave an opportunity to wash out all of the podophyllin which was free to move. After 15 to 20 minutes in 0.1

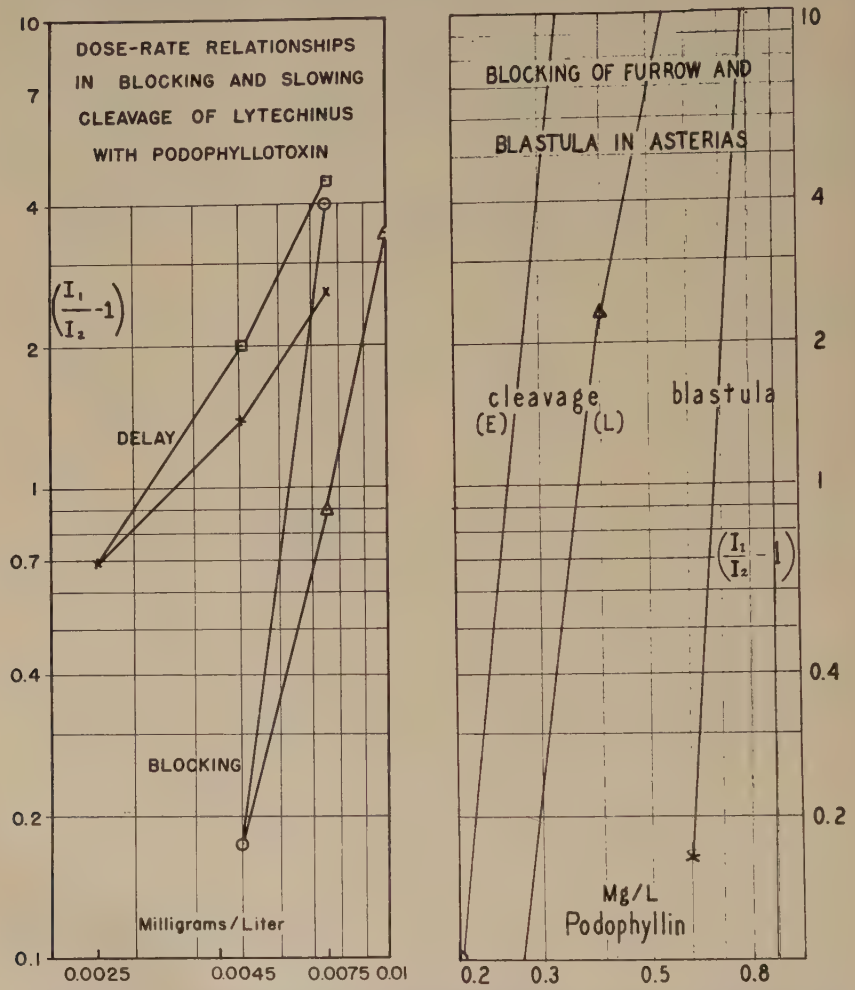


FIGURE 10. Abscissae are log dose in mg./L. Ordinates are ratio of inhibited to noninhibited (control-treated/treated) where control and treated are measured as percentage of cleavage, percentage of blastulae, or reciprocals of time to 50 per cent cleavage. *Lytechinus:* Blocking of normal cleavage ⊙; of abnormal cleavage △ (see text). Delay of first cleavage ×; of second cleavage ⊡. *Asterias:* Blocking of cleavage, exposure (E) 85 minutes, and (L) 33 minutes before control 50 per cent cleavage △. Blocking of blastulation, exposure 85 minutes before control 50 per cent cleavage ×.

mg./L. podophyllin, eggs were passed through several large volumes of water over a period of 15 minutes to 3.25 hours. The best subsequent development was a few irregular cleavages. Asters were sometimes visible in the eggs. Untreated eggs washed for four hours developed into normal gastrulae.

B. *Podophyllotoxin.* 1. *Exposure 11 to 12 Minutes After Fertilization.* (a)

Scale of Effects. No effect was observed at 0.001 mg./L. Cleavage was retarded at 0.0025–0.005 mg./L. Death occurred before gastrulation. Retardation and regression, or an incomplete block was observed at 0.0075–0.01mg./L. A complete block of cleavage was observed at 0.1 mg./L.

(b) *Dose-effect Relationships.* The relations of retardation and blocking to concentration are graphed in FIGURE 10, following the system used by Johnson in his review[22] and in earlier papers analysing dose-rate relationships in enzyme systems. Log dose is the abscissa and the ordinate is the log of the ratio between the affected portion of the population (control per cent − treated per cent) to the unaffected portion (treated per cent). This is expressed as $\left(\frac{I_1 - I_2}{I_2}\right)$ and simplified to $\left(\frac{I_1}{I_2} - 1\right)$. Measurements were percentage of eggs cleaved and the reciprocal of time to 50 per cent cleavage. The slope for inhibition of cleavage depends upon one's choice of criteria for blocking.* Interpreting first cleavage strictly as including only normal segmentation occurring within the time of the first division cycle gave the steeper slope (circles). As we have seen previously with *Asterias*, however, eggs could accumulate mitotic figures until they were able to divide at some later division cycle. Such cleavages, abnormal in morphology as well as mechanism, gave a less steep curve (triangles). Regression of furrows at a threshold dose was a common occurrence. These were not counted as cleavage. In either case, doubling the dose gave a hundredfold increase in effectiveness. Either cleavage required a quadrupling of the dose to increase percentage delay a hundred times. These graphs are based on single experiments, so no claim is made as to exact slope or straightness of the lines.

2. *Exposure Late in the Mitotic Cycle.* When treatment with 0.1 mg./L. was started at 37 and 27 minutes before control 50 per cent cleavage, fission was blocked. Exposure at eight minutes before control 50 per cent cleavage, just when the earliest eggs were furrowing, did not delay cleavage at 0.1 or even 1.0 mg./L. At the higher dose, however, cleavage reached only 76 per cent, then regressed to 60 per cent.

C. *Depodophyllotoxinized Resin.* Development was unaffected at 0.01 mg./L., while 0.1 mg./L. blocked segmentation if added 49, 37, or 27 minutes before control 50 per cent cleavage.

D. *Quercetin.* 1. *Stock Solution of $\frac{1}{4}$ per cent in Ethanol.* At 10 mg./L., there was an insignificant delay of cleavage, and at 25 mg./L., there was definite delay, with subsequent disorganization and death. Division was blocked completely at 100 mg./L., but this contained four per cent alcohol, and two per cent is sufficient to retard segmentation.

2. *Aqueous Stock Suspension.* The stock suspension of 100 mg./L. or its dilution to one-half or one-fourth were equally potent, all being saturated. These delayed the first two cleavages three to 20 minutes, but permitted continued development. In one experiment, the embryos took a centrolecithal pattern, with undivided cytoplasm surrounded by a layer of ciliated cells, but three attempts to duplicate these ontogenocopies were unsucessful.

3. *Aged Aqueous Solution.* Quercetin in fresh aqueous solutions is yellow

* See footnote, p. 1465

but begins to turn pink in a few hours. The pink supernatant from a saturated suspension which had been left at room temperature for 24 hours was found to delay the first cleavage 35 minutes and the second over an hour and to prevent development beyond irregular cell masses.

E. *Colchicine.* With exposure beginning 11 minutes after fertilization, 1 mg./L. did not delay the first or second cleavage but stopped development at the 32 to 64 cell stage. Ten mg./L. inhibited cleavage completely.

F. *Lytechinus: Comparisons.* At 0.04 to 0.1 mg./L. of podophyllin, cleavage was blocked, giving rise to karyomeres which could grow or dissolve and reappear. There was delayed surface deformation like that seen in other species. A series of continuous and short exposures, beginning at different times, revealed that sensitivity of the first mitotic cycle to podophyllin ended at metaphase.

Eggs placed in a concentration of podophyllin high enough to block cleavage, and then fertilized, were able to develop radial systems resembling rudimentary asters, or lines of flow around the male pronucleus. This "aster" did not function normally, in that the pronuclei remained separate in most ova. These continuously exposed eggs did not develop any farther. Eggs exposed for 15 to 20 minutes to 0.1 mg./L. and then fertilized in podophyllin-free sea water attained only a few irregular cleavages.

Podophyllotoxin was about ten times as effective as podophyllin. Comparison of dose and effect showed about a hundredfold increased inhibition for a doubling of concentration. Depodophyllotoxinized resin was as active as podophyllin. Results with late exposure could be superimposed upon those of the podophyllin series, and are, therefore, consistent with the same pattern of insensitive period.

A saturated solution of quercetin delayed cleavage. Aging a sea-water suspension for 24 hours increased its toxicity to the extent that it could block later segmentations.

Chromodoris

The egg of this sea slug is a beautiful cinnabar, a pleasure to watch as it progresses through its precise spiral cleavage. It is opaque, however, and is fertilized within the snail, which limits its usefulness for observation of cytological detail and for accurate timing. Moreover, the jelly envelope appears to retard penetration of chemicals. The treatments listed below began after the second cleavage, two hours before the third cleavage.

Podophyllin. No effect was observed at a concentration of 0.001 mg./L. At 0.01 mg./L., a block occurred before third cleavage.

Podophyllotoxin. No effect was observed at a concentration of 0.001 mg./L. At 0.01 mg./L., a block occurred during third cleavage.

Depodophyllotoxinized Resin. No effect was observed at a concentration of 0.001 mg./L. At 0.01 mg./L., a block occurred during third cleavage.

Colchicine. No effect was observed at 0.1 to 0.5 mg./L. Retardation resulted at 0.5 mg./L. At 1.0 to 2.0 mg./L., a block occurred after one or two cleavages. A complete block resulted at 10 mg./L.

Discussion

The sources of data drawn upon in the discussion are indicated by the letters and numbers of headings A2, B3a, *etc.* under *Results*.

Comparisons: Sensitivity of Different Species. The six species tested in this series exhibit as much as a tenfold difference in average susceptibility to each drug, but the relative potencies of the different drugs are about the same for all species (TABLE 4). The species averages, however, differ no more than do results from single tests on eggs from different females of the same species. Allowance must be made also for the less effective supernatant from an aqueous suspension used in the experiments with *Asterias*, *Arbacia*, and *Echinarachnius*.

Viehoever and Mack[39] reported toxicity of picropodophyllin and podophyllic acid less than that of podophyllotoxin when *Daphnia* was the test animal.

The Active Component of Podophyllin. Inasmuch as podophyllotoxin comprises up to half the crude podophyllin resin,[16, 40] is as active as podophyllin,

TABLE 4
CONCENTRATION RANGES AFFECTING CLEAVAGE*

	Pphyllin	*Ptoxin*	*Dtox resin*	*Quercetin*	*Colchicine*
Asterias	0.2–6	<0.01–0.01	—	1–10	1–10
Arbacia	0.4–6	0.05–0.6	—	0.2–40	10–80
Tripneustes	0.0005–1.5	0.0005–0.1	0.001–0.04	12.5–>sat.	1–40
Lytechinus	0.04–0.1	0.0025–0.1	>0.01–0.1	10–>sat.	1–10
Echinarachnius	0.1–1	0.01–0.04	—	10–20	—
Chromodoris	>0.001–0.01	>0.001–0.01	>0.001–0.1	—	0.5–10

* The smaller dose is the lowest concentration retarding division; the larger is the lowest concentration blocking division. in mg./L.

and induces the same physiological and structural responses, it was at first assumed that podophyllotoxin alone was responsible for the action of podophyllin. It came as a surprise to find that the resin from which the podophyllotoxin has been removed is just as active as either podophyllin or podophyllotoxin. In both *Tripneustes* and *Lytechinus*, however, the cytological events in depodophyllotoxinized resin are somewhat different. Karyomeres do not appear as quickly or as often, and do not pass through conspicuous nuclear cycles. The achromatic figure of *Tripneustes* persists longer than in podophyllin.

Parallel situations have arisen with other species. Philips, Chenoweth, and Hunt[32] found that podophyllin residues, after podophyllotoxin extraction, were as toxic as podophyllin or more so.

This applies to mitotic blocking, as well as to general toxicity in the mammal. Acetocarmine smears of crypts from the upper small intestine of weanling rats revealed that injections of podophyllotoxin in the amount of 12.5 mg./Kg. body weight trebled or quadrupled the mitotic count at the end of six hours. Only metaphases and pycnoses increased. The same dose of podophyllin gave identical results. Depodophyllotoxinized resin

was not quite as effective, but the chloroform-soluble fraction of crude podophyllotoxin was in turn fully as active at 12.5 mg./Kg. as were podophyllin and podophyllotoxin. The picture was comparable to that produced by colchicine at 1.6 mg./Kg. (Biesele, unpublished).

Hartwell[18] has isolated α-peltatin, an isomer of podophyllotoxin, from podophyllin, but no information is available as to its place in the above picture.

With *Tetrahymena*, a free-living ciliate, podophyllotoxin was less effective than podophyllin in slowing proliferation, although both were more effective than colchicine. Quercetin was five times as effective as podophyllin in retarding multiplication, or killing this protozoan. At 50 mg./L., proliferation was brought nearly to a standstill, and 250 mg./L. killed off most of the population (Ormsbee, unpublished data).

In disher containing *Asterias* eggs which were disintegrating as a result of poisoning with 100 mg./L. of podophyllin, it was noticed that various protozoa had increased in numbers. It may well be that mitosis in protozoa is as peculiar chemically as it is morphologically.

The remarkable ability of quercetin to retard cleavage for hours without killing the eggs does not fit the picture of podophyllin activity in the range of mitotic stasis. While the curve of podophyllin activity approaches that of quercetin at lower concentrations (FIGURE 2), the dilution of podophyllin is 1:1000, well below the threshold of quercetin, even assuming the stock solution to be saturated. Quercetin evokes interesting cytological events, but they are distinct from those of podophyllin. An antibiotic activity of quercetin has been reported by Andersen and Berry.[1]

Mitotic and Therapeutic Potencies. Condyloma. It has not been established that podophyllin heals the condyloma acuminatum by blocking mitoses. Sullivan and King[36] believe the action is exerted directly upon the cells, but find a type of degeneration which cannot be related directly to mitotic disruption. They do find intermediate stages, however, between this type of damaged cell and cells in which there has been a definite mitotic block[26]. The cures effected by colchicine point toward a mitotic route of action, and it might be suggested that the milder cytological effects of colchicine on skin and condylomata could be duplicated with weaker podophyllin.

The effective component would seem to be podophyllotoxin, and not only because of its preponderance in podophyllin[16, 40] and the similarity of their chemistry and of their effects on skin.[35] As Dr. Sullivan has pointed out, picropodophyllin, podophyllic acid, and quercetin did not have an effect on human skin comparable to that of podophyllotoxin.

Tissue Culture. The problem of finding the component or derivative of podophyllin which exerts a selective effect on tissue cultures is doubly engaging. The efficacy of the original serum—if podophyllin did endow it with such remarkable properties—and the better selective action of podophyllin, as against podophyllotoxin,[31] point to an additional active or potentiating factor. Podophyllotoxin was more toxic and had a narrower threshold than podophyllin.

Mouse Cancer in vivo. Hartwell and Shear[19] report that podophyllin,

podophyllotoxin, and α-peltatin all produced severe damage to sarcoma 37, while picropodophyllin and quercetin were negative at much higher doses.

Results obtained by others at the Sloan-Kettering Institute similarly gave picropodophyllin and quercetin, as well as rutin, a negative rating with *in vitro* exposure. For this test, cubes of S-180 were exposed at 4°C for 24 hours and then transplanted to mice. Pieces left untreated in Locke-Ringer and pieces exposed to the above compounds at saturation produced tumors. Some explants, exposed to a suspension of podophyllotoxin (100 mg./L.), were killed, and all of those exposed to a 1:256 dilution of saturated aqueous extract of podophyllin were nonviable.

Sarcoma 180 growing in mice also responded better to the crude podophyllin than to podophyllin "derivatives." Mice with four-day tumors were injected intraperitoneally once a day with 0.1 ml. of a 100 g./L. suspension of podophyllin in horse serum. Tumor growth was consistently diminished, but remissions were not significantly increased. Daily injections of podophyllotoxin at 2 mg./Kg. body weight as a gum acacia suspension slowed tumor growth, but less consistently than did podophyllin (Stock and Sugiura, unpublished).

Injection of sublethal concentrations of podophyllin into mice carrying spontaneous mammary carcinoma or five-day Sarcoma 180 resulted in a slowing of growth and even regression of tumors in some animals. It was not possible to eliminate the tumor in a significant number of animals, as judged by histological sections at autopsy (Rhoads, unpublished).

Podophyllin was also tested in doses of 15 to 30 mg./Kg. three times weekly for ten doses against transplanted leukemias AK 1394 and AK 9417 in mice, by the technique described previously.[8] Occasionally, some slight increase in survival time was noted, but this effect was definitely much less than that noted with the standard nitrogen mustards and, therefore, was not considered significant (Burchenal, unpublished).

The growth, histological appearance, and transplantability of the mouse Sarcoma 180 grown on the chorioallantoic membrane of the 12-day chick embryo[24] was not significantly altered by the yolk sac injections of the maximum tolerated doses of podophyllin, podophyllotoxin, podophyllin detoxified by heating it in an alkaline solution, and quercetin. The LD_{50} of these substances in the 12-day egg were as follows: podophyllin, 0.005 mg./egg; podophyllotoxin, 0.0012 mg./egg; heated podophyllin, 3.0 mg./egg; and quercetin, 10 to 20 mg./egg (Karnofsky, unpublished).

A Proposed Mechanism of Podophyllin Action. Selectivity of Podophyllin. The peculiar affinity shown by podophyllin for the mitotic figure is worth reviewing. Processes which proceed at concentrations greater than those which destroy the achromatic figure are: (1) normal, prompt lifting of the fertilization membrane (*Arbacia* A-1); (2) persistence of the meiotic cycle (dissolving of karyomeres from blocked maturation) (*Asterias* A-2a); (3) stimulation of a karyomere nuclear cycle by fertilization (*Asterias* A-2b.); (4) response of karyomeres and pronuclei to parthenogenetic (?) activation (*Asterias* A-2a, A-2b, A-2c, B. *Lytechinus* A-1); (5) ability of juxtaposed karyomeres to fuse into a single nucleus following prolonged individuality

(*Asterias* A-2c); (6) appearance within this nucleus of a single large nucleolus, evidence of a corresponding fusion of nucleoli (*Asterias* A-2c); (7) continued nuclear syntheses as revealed by karyomere growth, by production of nucleoli, and by increase in number of karyomeres following nuclear cycles. (*Arbacia* A-2, B-1, *Asterias* A-2b, A-2c, *Tripneustes* A-2, B, C, D); (8) sperm head hydration and fusion with the female pronucleus or formation of karyomeres (*Arbacia* A-1, *Asterias* A-2d, *Lytechinus* A-2, B-2); (9) formation and completion of the furrow when exposure is begun just before cleavage (*Arbacia* B-2, *Echinarachnius* B-2, *Asterias* A-1b, *Lytechinis* A-2a, A-2b); and (10) lack of effect on processes not requiring cell division.

In connection with the latter, (10), bipennaria larvae, adult starfish, and *Fundulus* were placed in 100 mg./L. podophyllin and remained active for hours. Diatoms and protozoa also moved actively among poisoned eggs.

Tolerance of these other processes could be exceeded. At 500 to 2000 mg./L., it was noted that karyomeres grew slowly and did not enter into nuclear cycles. Pronuclei were less susceptible to parthenogenetic activation. The very delayed cleavage that poisoned eggs undergo was more extensive if eggs were washed free of podophyllin. In fact, podophyllin in high concentrations took on a preservative function. Cells that were only partly poisoned cytolysed, apparently as a result of imbalance in the surviving activities. Eggs that had received an overdose remained intact.

Sequence of Events in the Mitotic Block. In trying to understand what is happening in the poisoned egg, one is confronted with the old problem of evaluating function from observation of structure. The two most transparent eggs gave discrepant answers. In *Echinarachnius* (A, B-1), the asters faded before cleavage began at threshold concentrations, while in *Lytechinus* (A-2), astral rays were detected in eggs which could not divide. Perhaps it merely means that the cytoplasm of *Lytechinus* is sufficiently clear to permit better observation of the aster remnant.

Nor was there strict correlation between the transporting power of the spindle and cytokinesis. Some eggs that did not cleave had daughter nuclei separated the full distance (*Asterias* A-1b), while some that did divide had nuclei close to the membrane (*Lytechinus* A-1c).

One generalization can be made: the only visible effect of podophyllin is destruction of the achromatic figure, and if this occurs early enough, cytokinesis is averted.

Dose-effect Relationship. Not only is there a sharp limit as to the time at which podophyllin can affect the subsequent segmentation, there is also a narrow limit of threshold concentrations. In FIGURE 10, plotting log dose against log ratio of inhibition illustrates the hundredfold increase of inhibition with a doubling of the concentration, whether the process is cleavage or blastulation. Data from *Arbacia* (A-2, B-1), *Echinarachnius* (A, B-1), and *Tripneustes* (A-1, B-1), while less complete, are entirely consistent with these curves. Whatever chain of events is involved, the end result of such an efficient ratio is the inactivation of several molecules of substrate by one molecule of poison. The point of action would presumably be one of

the enzymes which has a very rapid turnover during the time that the achromatic apparatus is being prepared and maintained.

The enzyme involved must be associated with mitosis in a special way. It can be inactivated by exposing mature ova before fertilization, while the pronucleus remains intact (*Arbacia* A-1, *Lytechinus* A-2). Thorough washing, however, weakens its influence only to a limited degree. Upon fertilization, then, every visible process which does not depend upon the spindle or aster proceeds unaltered. This weighs heavily in favor of an enzyme or system specific for building and sustaining the achromatic figure.

Alternative considerations are possible. Conceivably, the podophyllin could be bound by some relatively inactive component of the cell and kept available until the metabolic configuration becomes uniquely susceptible to the action of podophyllin. Either way, the specificity of podophyllin is not vitiated. It blocks a special process, whether that process results from the activity of a single special enzyme or from the joint activities of general enzymes. Metabolic and cytochemical studies are needed to determine whether the activities blocked by podophyllin are really as few as would appear from direct cytological observations.

Determination of the Furrow. Insensitive Period. We have seen that beginning exposure to podophyllin or podophyllotoxin, at about the time of metaphase or later, does not retard the cleavage of that division cycle (*Arbacia* B-2, *Echinarachnius* B-2, *Asterias* A-1b, *Lytechinus* A-2a). Short exposures show that enough podophyllin had penetrated between the beginning of the insensitive period and the beginning of cytokinesis to affect cleavage, because the next division is retarded and abnormal (*Lytechinus* A-2b). Yet, although no furrow forms, and although the furrow primordium is at the surface where podophyllin should reach it first and in highest concentration, the first cytoplasmic division is not influenced.

The most convincing demonstration of the unswerving perseverance of a furrow in its predetermined position comes from the centrifugation studies of Dr. E. B. Harvey. If the spindle, or even the intact prophase nucleus, is displaced, cleavage comes in the original equatorial position.[20]

The furrow, then, is determined before it appears. One logically looks to the obvious event preceding furrow formation—the anaphase of karyokinesis. The literature, which cannot be reviewed here, is full of evidence to show that destruction, weakening, displacement, or distortion of the mitotic figure results in suppression or distortion of the furrow. An active concentration of podophyllin always diminished or destroyed the mitotic apparatus. While we cannot show an unequivocal causal connection between achromatic figure and spindle, the weight of coincidence is heavy.

We must also consider the possibility that the refractoriness of the segmentation process arises from the nature of the furrow itself. Perhaps podophyllin acts directly upon the furrow, but acts slowly, and is not able to make itself felt in the space of ten minutes. A phenomenon which weighs against this supposition is discussed in the next section.

Extreme Delay of Cleavage. In all the Echinoderm eggs studied, eggs continuously exposed to strong podophyllin eventually form one or more furrows (*Arbacia* B-1; *Asterias* A-1a, A-2b, A-2c, B; *Tripneustes* B; *Lytechi-*

nus A-1, A-2c, (PLATE 2, 10), and sometimes divide completely, particularly when fertilized (PLATE 2, 11). Apart from their abnormal position, these clefts appear to be true furrows, as judged by their external and internal (PLATE 2, 9) morphology and by their ability to divide the egg into progressively smaller portions without evidence of any cytolytic fragmentation. Analysis of their behavior at high pressures (Marsland) would help clarify their status.

Such dividing eggs, it must be remembered, were continuously exposed to a blocking dose of podophyllin. There can be no question here of a refractory period arising from slowness of response of the furrow. It brings us back to the original working hypothesis, that an event preceding cytokinesis determines the furrow.

Relationship Between Chromatin and the Furrow. The late cleavage described above never occurs in *Asterias* eggs retaining the germinal vesicle, or in the other eggs when they have not been fertilized. Eggs continuously exposed to a blocking dose, but fertilized, cleave better than unfertilized eggs. It is not always possible to establish a correlation between furrowing and dissolving of karyomeres, but, in a few experiments, this is a salient feature. One set of *Asterias* eggs, for example, was under examination 11 hours after the beginning of the test. Within ten minutes, the karyomeres disappeared in most of the eggs. Shortly thereafter, the surface was divided simultaneously into a layer of tiny spherules which gave the eggs a spurious resemblance to the control blastulae.

A better example can be drawn from *Lytechinus* eggs under exposure to 0.1 mg./L. podophyllin for four hours. There was a paroxysm of bulging in most of the eggs. Few of these eggs had karyomeres, although there were karyomeres in most of the eggs which did not show activity at the surface, and, at the previous observation, all the eggs contained karyomeres. Apparently, any nucleus, upon dissolving, supplies something necessary or helpful to cleavage.

The spatial relationships between furrows and chromatin are also revealing. Furrows in the greatly retarded *Asterias* eggs always have their origin near the polar body, whenever the polar body can be seen (PLATE 2, 9). Sections of eggs with multiple furrows reveal scattered chromatin or tiny distorted mitotic figures near many of the furrows (*Asterias* A-1a).

Furrow activity maintains a relationship with chromatin and not with the asters. In place of the classical concept of the mechanical activation of the furrow by the asters (a theory made temptingly reasonable by the excellent work of the Dans[15]), it is preferable to substitute a concept of chemical induction of the furrow, at least to fit the findings of the present research with podophyllin.

The idea of chemical evocation of segmentation is not new, although it was generated *de novo* in the mind of the author in attempting collation of the above results. Stimulating ideas about a chemical furrow-evoker and other ideas consonant with such evocation have been expressed elsewhere in this monograph, and they will be related to a working hypothesis in the final section. In plant meristematic cells at least, the process of separation of

the daughter cytosomes appears to be chemical from beginning to end. Re-examination of the literature is in progress, with a view to finding data or theories in line with this hypothesis, but at present only one can be quoted. Dalcq[13] and Dalcq and Simon[14] found a revealing situation in irradiation, trypaflavine, and androgenesis experiments with the frog's egg. They found that furrow formation required a minimum of chromatin. Even a trypaflavine-inactivated sperm head could evoke a furrow, but an empty achromatic figure never did. Dalcq, however, conceives cytokinesis as a dual process—a chemical separation of the two cytosomes, followed by the mechanical intrusion of the furrow.

Regression of the Furrow. An important phenomenon deserving of further attention is the disappearance of furrows from eggs exposed to threshold doses (*Arbacia* A-2, B-1; *Echinarachnius* A; *Asterias* A-1a; *Tripneustes* A-2; *Lytechinus* B-1a) or exposed just at the end of the podophyllin-sensitive period (*Echinarachnius* B-2, *Lytechinus* A-2a, A-2b, B-2). In the extreme case, nearly all the eggs appear to be two-celled at one moment. The furrows then become progressively more shallow and eventually disappear. Whether this process results from fusion of the apposed furrow walls or from actual withdrawal of the furrow, cannot be decided for certain from the present observations. Withdrawal seems the more probable, for the cleavage walls remain stable in eggs that complete cleavage during the first cycle or in eggs that successfully cleave at a later time.

Be that as it may, regression of the furrow reveals a critical stage just at the completion of cytokinesis. It also negates theories invoking surface tension as the incising force following elongation of the egg, unless one can believe in a violent local switch to negative tension.

Chemical Induction of the Furrow: A Hypothesis. To tie together the facts and suppositions discussed above, we can trace the presumed course of events in a mitotic cycle correlating the different steps with ideas presented by others in this monograph.

(1) The nuclear membrane dissolves, releasing nuclear sap at the end of prophase. Dr. Chalkley has explained how material rich in sulfhydryl groups is released by the nucleus and suggested the possibility that this may be involved in starting cytoplasmic fission.

(2) The furrow determiner from the nucleus is carried in a nearly symmetrical pattern to the cell cortex. Dr. Conklin has described how the nuclear material gathers around the centrosomes, and then is transported through the asters to the cell surface. In other eggs, Dr. Chambers has seen currents flowing toward the equator, contributing to the inward growth of the furrow. The extreme centrifugal acceleration sustained by *Ascaris* eggs is sufficient to displace a furrow-forming substance, as Dr. Beams has explained. He has already brought the evidence from the work of Heilbrunn and of Moore to the support of this possibility. Dr. E. B. Harvey's success in producing parthenogenetic merogons constitutes an obstacle. We might propose that, since the ovum has undergone maturation, nuclear sap has already been released by the germinal vesicle, and, therefore, while the eggs lack chromatin, they do not lack nuclear substance.

(3) The determiner from the nucleus then sets the furrow in motion.

Progress of the furrow can be thought of as growth, as Dr. Chambers has described it, or as contraction, as Dr. Lewis believes. In the first case, the fission evoker would be an activator of contractile elements already within the furrow. In the second case, the inductor substance would be actually built into the furrow, as conceived by Dr. Kopac.

In either case, the furrow is a distinct, functioning entity, which Dr. Marsland is able to identify as a gel band even before indentation starts. If the furrow is autonomous once it has started, as it seems to be in the podophyllin experiments, contraction more easily fits the concepts of self-perpetuating systems, but an autocatalytic growth of the furrow gel is equally possible, and we can draw on the autocatalytic clotting of blood as a model.

When podophyllin has crippled or destroyed the achromatic figure, the furrow determiner reaches the cortex late and in an irregular pattern, inducing very delayed, abnormal cleavage. This cleavage can be improved by adding more determiner *via* fertilization.

(4) When the furrow is nearly completed, a process separate from those which caused the furrow to advance is required to complete the separation of the blastomeres. Have contractile elements in the furrow reached their minimal length at this point? Dr. Chambers and Dr. Lewis have emphasized the persistence of a cytoplasmic column between daughter cells. Conceivably, spindle material is essential for the final break, and, when this has been dispersed as a result of podophyllin poisoning, the cytoplasmic connection persists. Then, when the furrow relaxes at the end of its cycle, the cytoplasmic bridge widens and the blastomeres are joined as one cell again.

Summary

(1) The substances studied were colchicine, podophyllin, podophyllotoxin, picropodophyllin, podophyllic acid, depodophyllotoxinized podophyllin, and two fractions of depodophyllotoxinized crude podophyllotoxin. The effective ranges are shown in TABLE 4. The range for the chloroform-soluble fraction of depodophyllotoxinized crude podophyllotoxin was 0.0005 to 0.05 mg./L., and for the chloroform-insoluble fraction, >0.005 to 0.05 mg./L., when tested on *Tripneustes* eggs.

(2) Eggs of *Arbacia punctulata*, *Asterias forbesii*, *Echinarachnius parma*, *Tripneustes esculentus*, *Lytechinus variegatus* and *Chromodoris sp.* revealed as much as a tenfold difference in susceptibility of different species.

(3) The cytological effects of podophyllin, podophyllotoxin, picropodophyllin, podophyllic acid, and colchicine were essentially alike. These resulted from destruction or crippling of the achromatic meiotic or mitotic figure.

(4) Those aspects of fertilization and mitosis which do not depend on the achromatic figure were not visibly affected. The most important were: lifting of the fertilization membrane and hydration of the sperm head; formation, growth, and fusion of karyomeres; cyclic changes in the karyomeres as the result of internal rhythms and external stimuli; and completion of cleavage once the furrow had been determined.

(5) Resistance of the furrow to direct action of podophyllin was revealed

by beginning exposure at successively later times. Once the eggs had passed metaphase, cleavage could not be altered.

(6) At threshold concentrations, or with exposures beginning near metaphase, many furrows nearly divided the egg and then regressed.

(7) There was delayed furrowing and division in blocked, continuously exposed eggs at 10 to 24 hours. This occurred only in eggs where there had been at least one nuclear or pronuclear breakdown. There was better correlation of furrows with chromosomes than with asters.

(8) The dose-effect relationships show that the efficacy of podophyllin and podophyllotoxin increased about a hundredfold for each doubling of concentration.

(9) Exposing the eggs, and then washing them in several changes of sea water before fertilization, permitted only a few, delayed, abnormal cleavages.

(10) Podophyllotoxin was the most active pure substance obtained from podophyllin, but unidentified components of some fractions were equally potent.

(11) One such active component or combination present in depodophyllotoxinized resin produced effects different from those of podophyllin and podophyllotoxin.

(12) Quercetin in large doses did not destroy asters and spindle, and retarded division several hundred per cent without blocking any stage of development, whereas all the other substances blocked early cleavages in some or all the eggs before retardation reached 100 per cent (*i.e.*, doubling of the time between fertilization and cleavage).

(13) The results have been explained by assuming that a furrow-organizer is released from the nucleus at the end of prophase and is distributed to the equatorial region by the achromatic figure. It then causes the furrow to form and to progress through the egg. When podophyllin incapacitates the achromatic figure, the furrow-organizer reaches the cortex late, and in an irregular pattern, causing delayed, irregular furrowing. Closing off the final connecting strand of cytoplasm is a distinct process, separately dependent on the presence of the spindle.

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Bibliography

1. Andersen, A. A. & J. A. Berry. 1947. Effect of flavonols on *Clostridium botulinum*. Science **106:** 644–645.

2. Attree, G. F. & A. G. Perkin. 1927. The position of the sugar nucleus in the quercetin glucosides. J. Chem. Soc. **1927:** 234–240.
3. Beams, H. W. & T. C. Evans. 1940. Some effects of colchicine upon the first cleavage in *Arbacia punctulata*. Biol. Bull. **79:** 188–198.
4. Belkin, M. 1947. Effect of podophyllin on transplanted mouse tumors. Fed. Proc. **6:** 308.
5. Belkin, M. 1948. Effect of podophyllin on transplanted mouse tumors. J. Pharm. Exp. Ther. **93:** 18–25.
6. Borsche, W. & J. Niemann. 1932. Über Podophyllin. Justus Liebigs Ann. Chem. **494:** 126–142.
7. Borsche, W. & J. Niemann. 1932. Über Podophyllin II. Justus Liebigs Ann. Chem. **499:** 59–76.
8. Burchenal, J. H., R. A. Lester, J. B. Riley, & C. P. Rhoads. 1948. Studies on the chemotherapy of leukemia. I. Effect of certain nitrogen mustards and carbamates on transmitted mouse leukemia. Cancer **1:** 399.
9. Chambers, E. L. 1939. The movement of the egg nucleus in relation to the sperm aster in the echinoderm egg. J. Exp. Biol. **16:** 409–424.
10. Cornman, I. 1947. The effects of podophyllin on the maturation and cleavage of the starfish egg. Fourth Internat. Cancer Res. Cong. Abstracts: 53.
11. Cornman, I. 1947. The effects of podophyllin on the maturation and cleavage of the starfish egg. Biol. Bull. **93:** 214.
12. Cornman, I. 1947. Inhibition of *Arbacia* egg cleavage by podophyllotoxin and related substances. Biol. Bull. **93:** 192.
13. Dalcq, A. 1929. Le rôle dynamique des chromosomes dans la caryocinèse et la plasmodiérèse. C. R. Assoc. Anat. **24:** 176–192.
14. Dalcq, A. & S. Simon. 1930. Analyse de la segmentation de l'oeuf de grenouille par l'irradiation combinée des deux gamètes. C. R. Assoc. Anat. **25:** 104–120.
15. Dan, K. & J. C. Dan. 1947. Behavior of the cell surface during cleavage. VII. On the division mechanism of cells with excentric nuclei. Biol. Bull. **93:** 139–188.
16. Dohme & Kelly. 1904. J. A. Ph. A. **52:** 561. (Quoted in Viehoever and Mack.)
17. Fry, H. J. 1936. Studies of the mitotic figure. V. The time schedule of mitotic changes in developing *Arbacia* eggs. Biol. Bull. **70:** 89–99.
18. Hartwell, J. L. 1947. α-peltatin, a new compound isolated from *Podophyllum peltatum*. J. Am. Chem. Soc. **69:** 2918.
19. Hartwell, J. L. & M. J. Shear. 1947. Chemotherapy of cancer. Classes of compounds under investigation and active components of podophyllin. Cancer Res. **7:** 716–717.
20. Harvey, E. B. 1935. The mitotic figure and cleavage plane in the egg of *Parechinus microtuberculatus*, as influenced by centrifugal force. Biol. Bull. **69:** 287–297.
21. Heap, T. & R. Robinson. 1926. A synthesis of kaempferide and of isorhamnetin. J. Chem. Soc. **1926:** 2336–2344.
22. Johnson, F. H. 1947. Bacterial luminescence. Adv. Enzymol. **7:** 215–264.
23. Kaplan, I. W. 1942. *Condylomata acuminata*. New Orleans Med. Surg. J. **94:** 388–390.
24. Karnofsky, D. A., J. H. Burchenal, R. A. Ormsbee, I. Cornman, & C. P. Rhoads. 1947. Experimental observations on the use of the nitrogen mustards in the treatment of neoplastic disease. Approaches to Tumor Chemotherapy. A.A.A.S. 293–305.
25. King, L. S. & M. Sullivan. 1946. The similarity of the effect of podophyllin and colchicine and their use in the treatment of condylomata acuminata. Science **104:** 244–245.
26. King, L. S. & M. Sullivan. 1947. Effects of podophyllin and of colchicine on normal skin, on *condyloma acuminatum* and on *verruca vulgaris*. Arch. Path. **43:** 374–386.
27. MacCardle, R. C. & V. Downing. 1947. Histologic criteria for evaluating the capacity of chemical agents to produce damage rapidly in sarcoma 37. Cancer Res. **7:** 717.
28. MacCardle, R. C. & A. Perrault. 1947. Degeneration of cerebellar Purkinje cells in tumor-bearing animals after subcutaneous injection of podophyllin. Fourth Internat. Cancer Res. Cong. Abstracts: 52–53.
29. Nebel, B. R. & M. L. Ruttle. 1938. The cytological and genetical significance of colchicine. J. Hered. **29:** 3–9.
30. Ormsbee, R. A. & I. Cornman. 1947. The effect of podophyllin on tumor cells *in vitro*. Cancer Res. **7:** 717.
31. Ormsbee, R. A., I. Cornman, & R. E. Berger. 1947. Effect of podophyllin on tumor cells in tissue culture. Proc. Soc. Exper. Biol. & Med. **66:** 586–590.

32. Philips, F. S., M. B. Chenoweth, & C. C. Hunt. 1948. Studies on the toxicology of podophyllotoxin and related substances. Fed. Proc. **7:** 249.
33. Späth, E., F. Wessely, & E. Nadler. 1933. Zur Konstitution des Podophyllotoxins und Pikro-podophyllins. Ber. deut. chem. Ges. **66:** 125–130.
34. Sullivan, B. J. & H. I. Wechsler. 1947. The cytological effects of podophyllin. Science **105:** 433.
35. Sullivan, M. & K. Blanchard. 1947. Podophyllotoxin. Bull. Johns Hopkins Hosp. **81:** 65–67.
36. Sullivan, M. & L. S. King. 1947. Effects of resin of podophyllum on normal skin, *condylomata acuminata* and *verrucae vulgares*. Arch. Dermatol. Syph. **56:** 30–45.
37. Sullivan, T. D. 1947. Somatic chromosomes of pedigreed hybrid petunia. Bull. Torrey Bot. Club **74:** 453–475.
38. Theophrastus. Enquiry into plants and minor works on odours and weather signs.
39. Viehoever, A. & H. Mack. 1938. Biochemistry of May apple root (*Podophyllum peltatum*). I. J. Am. Pharm. Assn. **27:** 632–643.
40. Warren, L. E. 1927. Comparison of several processes for the assay of podophyllum. J. Assoc. Official Org. Chem. **10:** 272–280.
41. Wilbur, K. M. 1940. Effects of colchicine upon viscosity of the *Arbacia* egg. Proc. Soc. Exp. Biol. and Med. **45:** 696–700.
42. Zemplén, G. & G. Árpád. 1935. Konstitution und Synthese der Rutinose, der Biose des Rutins. Ber. deut. chem. Ges. **68:** 1318–1321.
43. King, L. S. 1948. Effects of podophyllin on mouse skin. I. Histologic sequence after a single dose. J. National Cancer Inst. **8:** 215–225. (Published after preparation of this manuscript.)
44. Anderson, C. R. 1946. Arch. Derm. Syph. **54:** 66; Beheran, H. R. & J. J. Kraves. 1945. Día. Méd. **17:** 1137–1138; Canavosio, A. E. 1946. Rev. san. mil., Buenos Aires **45:** 793–795; Cohen, E. L. 1946. Practitioner **156:** 133–134; Cordero, A. A. 1946. Rev. argent. dermatosif. **30:** 101–105; Culp, O. S. & I. W. Kaplan. 1944. Ann. Surg. **120:** 251–256; Culp, O. S., M. A. Magid, & I. W. Kaplan. 1944. J. Urol. **51:** 655–659; Finkle, T. H. & E. J. Frischwasser. 1947. J. Invest. Dermat. **8:** 199–201; Garnier, G. 1947. Paris méd. **37:** 50–51; Haber, H. 1945. Brit. J. Ven. Dis. **21:** 63–64; MacGregor, J. V. 1945. Brit. Med. J. **1:** 593–594; Michel, P. J. 1946. J. méd. Lyon. **27:** 567–570; Monzo, O. R., J. Kantt, & M. F. Couretot. 1946. Semana méd. **1:** 298–300; Reich, W. J., M. J. Nechtow, & M. R. Rubenstein. 1947. Am. J. Obst. & Gynec. **53:** 658–662; Ronchese, F. 1947. R. I. Med. J. **30:** 103–104; Serrano Camargo, M. 1945. Med. y Cir., Bogotá **9:** 188–190; Trolle, D. 1946. Nord Med. **31:** 1886–1888.

PLATE 1.

1. Living *Asterias* egg. Fertilized egg exposed to threshold concentration (0.6 mg./L.) of podophyllin during second maturation division. Cleavage was inhibited, but achromatic figures proliferated (*Results:* A 1 a). Those in egg at left appeared to be dividing (*cf.* photograph 2). Egg slightly flattened. 200 ×.

2–4. Harris hemalum-eosin sections of fertilized *Asterias* eggs placed in 1 mg./L. of podophyllin after completion of maturation (*Results:* A 1 a). Fixed four hours later, when the living eggs resembled those in 1 and 5. 2. Anastral anaphase. 1300 ×. 3. Two heavily stained irregular mitotic figures in the large blastomere. Vesicular nucleus in two of the small blastomeres. 400 ×. 4. Two clusters of chromatin masses at center and periphery of undivided egg. Egg at right undergoing multiple division. 300 ×.

5 and 6. Fertilized *Asterias* eggs placed in 0.6 mg./L. podophyllin after completion of maturation. Multiple cleavage three hours later. 5. Multiple achromatic figures and bulging. 6. Six minutes later, same field. 75 ×. (*Results:* A 1 a).

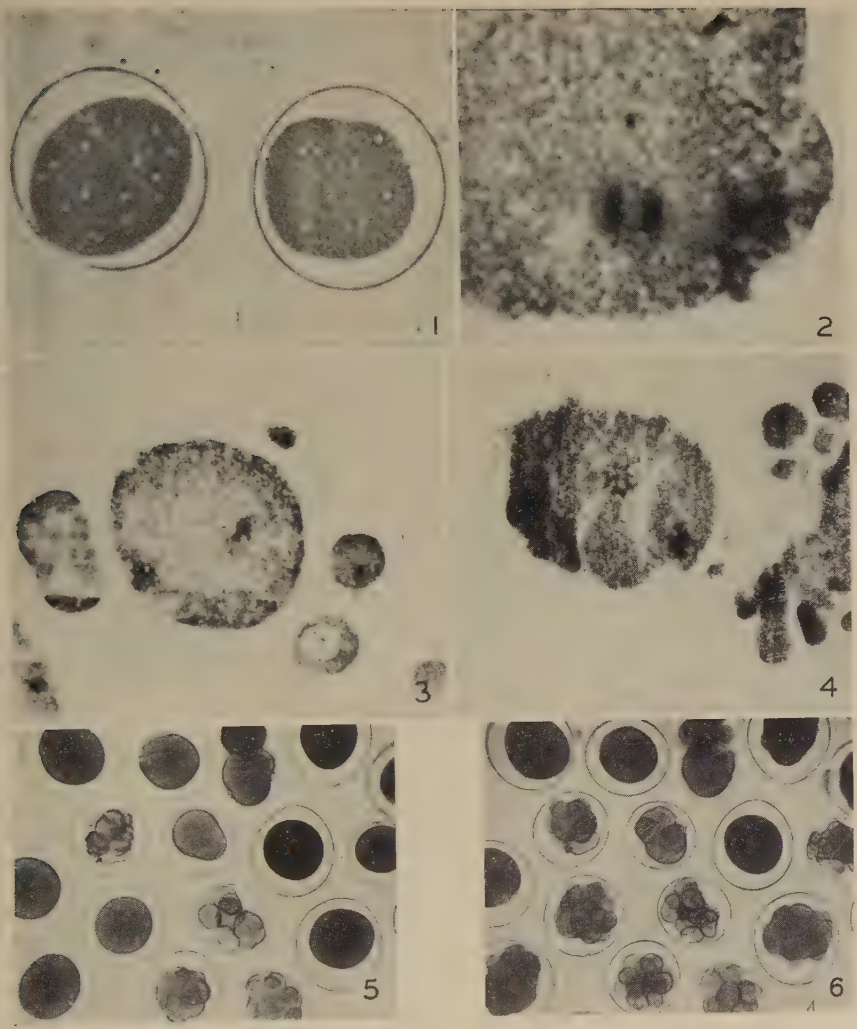
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PLATE 2.

7. Hemalum-eosin section of unfertilized *Asterias* egg placed in 100 mg./L. podophyllin during extrusion of first polar body. In the living eggs the spindle disappeared. Karyomeres appeared and, in turn, were replaced by an elongate hyaline body two hours after exposure began. Sections of samples fixed at two hours revealed strongly basophilic oval to fusiform structures, too dense to permit identification of any chromatin. 500 ×. (*Results:* A 2 b).

8–12. Unfertilized eggs exposed to 100 mg./L. podophyllin while germinal vesicle was still intact (*Results:* A 2 c). 8, 10, and 11. Continuous exposure. Fertilized at 3.5 hours. Fixed at 13 hours. One-sided (10) and unequal complete (11) division. Karyomeres in the whole egg (11) at 250 ×, and in the hemalum-eosin section (8) at 500 ×. The karyomeres are densely basophilic. 9. 30 minutes exposure. Unfertilized. Fixed at 8 hours. Section through one-sided furrow. H. and E. 400 ×. 12. 30 minutes exposure. Fertilized at 3.5 hours. Fixed at 8 hours. Irregular cleavage. 75 ×.

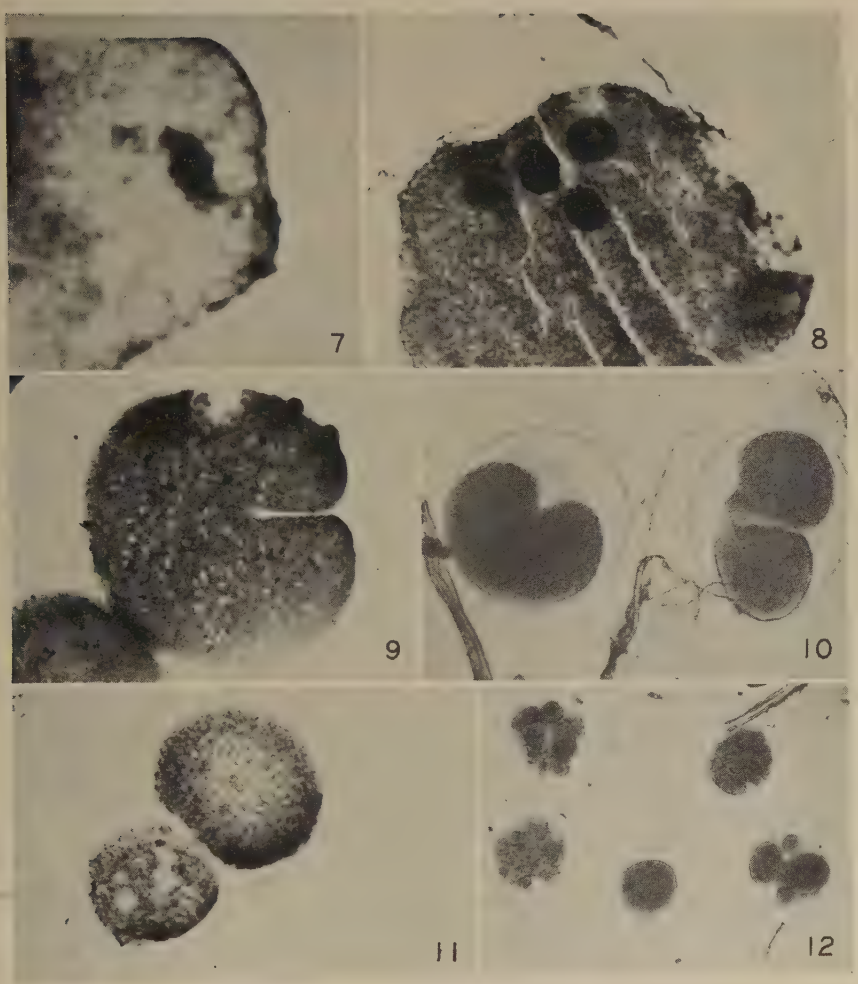
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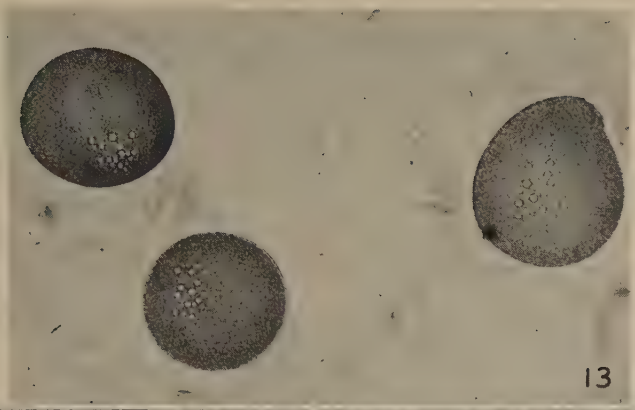
PLATE 3.

13–16. Living *Asterias* eggs. Unfertilized oöcytes exposed to 100 mg./L. podophyllin when the germinal vesicle had broken down in 52 per cent of the eggs (*Results:* A 2 c). 13. At four hours; 30 karyomeres were visible. Egg slightly flattened. 125 ×. 14. At seven hours, karyomeres in a compressed egg. 850 ×. 15. At nine hours. Karyomeres fusing in one egg. Flattened. 150 ×. 16. At 11 hours. Complete fusion of karyomeres. Nucleus (upper right) about $\frac{3}{4}$ diameter of germinal vesicle (lower left). Distinct nucleolus. 125 ×.

17. Living *Asterias* egg. Exposed to 100 mg./L. podophyllin during extrusion of second polar body (*Results:* A 2 a). Hypohaploid number of karyomeres visible at one hour. Egg flattened. 200 ×.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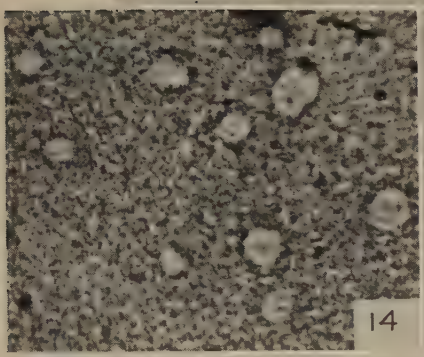
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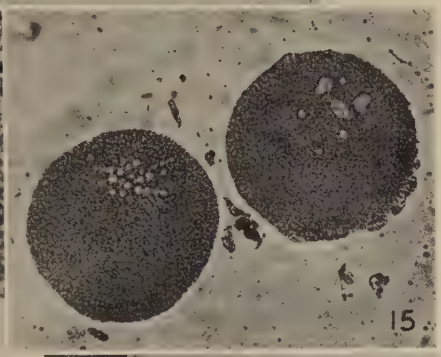
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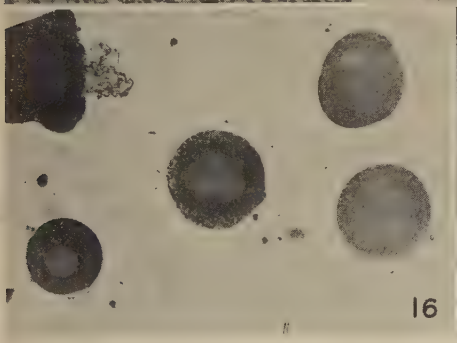
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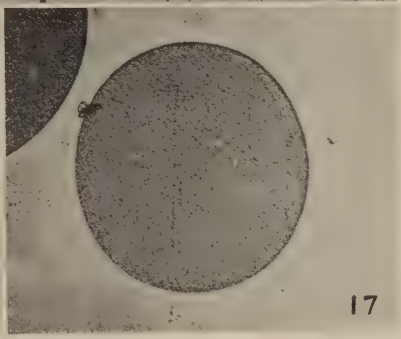
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EFFECTS OF MITOTIC INHIBITORS ON TUMOR CELLS

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Historical Account

More than 50 years ago, Fol (1876) experimentally induced multipolar mitosis and arrested the movement of chromosomes by subjecting sea-urchin eggs to low temperatures, and Oscar Hertwig (1878) showed that this condition may be produced by various toxic agents, such as strychnine and chloral hydrate. While multipolar and monocentric mitoses may occur spontaneously under natural circumstances, it was long ago discovered that such unusual phenomena can be produced experimentally by CO_2 (Herbst, 1887), phenyl urethane (Painter, 1916), ether (Wilson, E. B., 1901), mechanical injury (Boveri, 1901), and lack of oxygen (Demoor, 1895). All of these agents cause a diminished development or even a complete suppression of the achromatic figure, with subsequent failure of division. These early experiments demonstrated that the division of both cytosome and nucleus may be halted while that of the chromosomes and the central bodies steadily proceeds.

Wilson (1901) found that, when cytokinesis is sufficiently reduced by the action of ether, only slight separation of the daughter chromosomes occurs, and that they may form together a single nucleus. By the continuation of this narcotizing action, the number of both chromosomes and astral centers increases and the nucleus becomes larger. Thus arises a giant polyploid nucleus which, upon subsequent mitosis, develops a large number of chromosomes scattered among spindles associated with many division centers. Boveri felt that the phenomenon of mitosis involves two fundamentally independent processes, *i.e.*, the origin and transformation of the achromatic figure and the formation and division of the chromatic elements.

Mitotic activity can be reversibly inhibited by such agents as cold, acids, and anaesthetics. Ephrussi (1926) concluded that each phase of mitosis is inhibited at a particular temperature coefficient, that of the prophase being high and that of the anaphase movement being low. Mathews (1907) found that the application of quinine and KCN during mitosis leads to a disappearance of asters and spindle, but that the chromosomes are unaffected by these agents. Exposure to X rays does not affect mitosis, according to Strangeways and Hopwood (1926) and Henshaw (1940), whereas mitosis is prevented in the subsequent division after the cell has been irradiated during the interkinetic period.

X rays are said to induce stickiness of chromosomes, with lagging and abnormal distribution on the spindle. This is believed to be due to polymerization of the nucleic acid at the surface of the chromosome (Lea, 1947). Mitchell (1942) suggested that the delay in division after X radiation is due to an interference in the nucleic acid cycle, resulting in a failure of the conversion of ribonucleic acid to desoxyribonucleic acid. Ribonucleotides ac-

cumulate in the cytoplasm of cells after irradiation. Recently, Sparrow and Rosenfeld (1946) and Taylor, Greenstein, and Hollaender (1948) have shown that X irradiation of solutions of sodium salt of thymonucleic acid results in fragmentation of its long fibrous particles and polydispersion. Smith and Clowes (1924) found that, when the CO_2 tension of sea water is increased, the velocity of cell division in *Arbacia* is decreased, and that mitosis ceases altogether at a definite tension of the gas.

Multipolar and monocentric mitoses are not uncommon in animal and human tumors. Bizarre forms of abnormal mitotic figures with and without centrioles and astral rays occur, for example, in the cells of spontaneous and induced hepatomas, skin and gastric carcinomas, and sarcomas. The writer recently observed anastral mitosis in a human carotid body tumor. It is clear, however, that tumors, like normal tissues, grow primarily by typical mitosis, and that abnormal mitotic figures are of secondary origin. J. W. Wilson and Leduc (1948) observed multipolar mitoses in normal livers of mice.

Abnormal mitoses can be produced artificially in tumors by a large number of different chemical agents. At the National Cancer Institute, a group of investigators, including chemists, biologists, pharmacologists, physiologists, clinicians, and cytologists have been working in a cooperative project in the Chemotherapy Section to survey in a preliminary way the capacity of a number of chemical agents to produce necrosis or other changes in tumor cells at 8 to 48 hours after a single sublethal dose injected subcutaneously in the contralateral axilla of mice bearing sarcoma 37 implanted in the thigh muscles. Briefly, the effects of a chemical agent may be ascertained by observing reversible or irreversible phenoma such as (1) induced necrosis produced within a short period of time when it can be distinguished from old spontaneously derived necrosis, (2) mitotic aberrations, (3) changes in basophilia and acidophilia of cytoplasmic and nuclear components, (4) vacuolization, and (5) modifications of the stroma. Changes in intracellular minerals, Golgi substance, mitochondria, and the achromatic figure may also serve as valuable criteria, and the physical properties of certain cell constituents as determined by ultracentrifugation should also be investigated by chemically treated tumor cells. The present paper is part of this project, the preliminary reviews of which have been published (Shear, *et al.*, 1949; Downing, *et al.*, 1949; and MacCardle, 1949).

The following description deals chiefly with mitosis in tumor cells treated with an iodine-containing derivative of colchicine and with podophyllin. The changes induced by these mitotic inhibitors are compared with those produced by other agents such as anaesthetics, arsenic, trypaflavine, and lack of oxygen. An important problem is to ascertain the cytologically detectable mechanism by which a chemical agent may injure a tumor cell, and to determine whether such a mechanism may be characteristic of a particular chemical.

Effects of Chemicals on Mitosis in Tumor Cells

1. *N-acetyliodocolchinol Methyl Ether.* In mouse sarcoma 37, the typical metaphasic mitotic figure in fixed preparations constitutes about 40

small double chromosomes dispersed throughout the whole cross-sectional area of the middle of a conspicuous anastral spindle, the fibers of which terminate at either of its two division poles in end-zones of clear cytoplasm that may or may not be equipped with centrioles (FIGURES 1, 2). The dividing cell is always less basophilic and larger than its resting neighbor cells. In tumor cells teased in physiological saline and observed under phase-contrasting light, the chromosomes and spindle area are clearly visible, although no spindle fibers are discernible.

In sarcoma 37 cells, treated 24 hours previously *in vivo* with a single sublethal dose of 300 micrograms of N-acetyliodocolchinol methyl ether injected subcutaneously in tumor-bearing mice, mitosis is apparently stimulated and then arrested, usually in the metaphase but also in various ana-

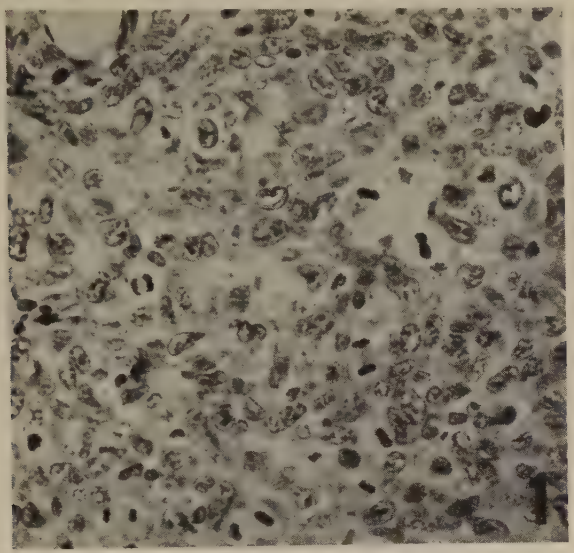

FIGURE 1. Control sarcoma 37. Zenker-formol. Haematoxylin/eosin. Low power.

phase and telophase stages of division (FIGURES 3–5). There are many multipolar mitoses in tumors treated with lower doses of this agent. In some of the cells arrested in metaphase with 300 micrograms of acetyliodocolchinol, no evidence of a spindle may be seen. In other cells, however, fragments of spindle fibers can be detected in sections prepared by Heidenhain's iron-alum haematoxylin method. Chromosomes arrested in metaphase position appear to be displaced around the periphery of a central sphere of material that is densely colored with haematoxylin. When observed in the living state in teased preparations, the chromosomes of such (previously *in vivo*) treated cells may be seen to be moving around the periphery of a clear glassy area, as though confined between the peripheral surface of a centrally located nuclear sphere and the wall of a thick cytoplasm constituting the remainder of the cell.

Microincineration of these treated cells arrested in metaphase reveals that the spindle area is clearly discernible by its rich deposit of flat white ash of

calcium and/or magnesium. The central nuclear sphere contains little white ash, but a varying amount of red iron oxide particles may be confined to this area. A comparison of treated and untreated microincinerated tumor cells may be seen in FIGURE 6. If the treated cells arrested in metaphase are first centrifuged at about 200,000 times gravity in a Beams ultracentrifuge, and then microincinerated, it may be seen that the entire central nuclear sphere, with its peripherally located chromosomes, can be moved through the cytoplasm as an intact body (FIGURE 7), whereas in untreated cells the chromosomes are evidently ejected from the spindle or moved along its axis. The centrifugal separation of the mineral ash in these treated cells probably

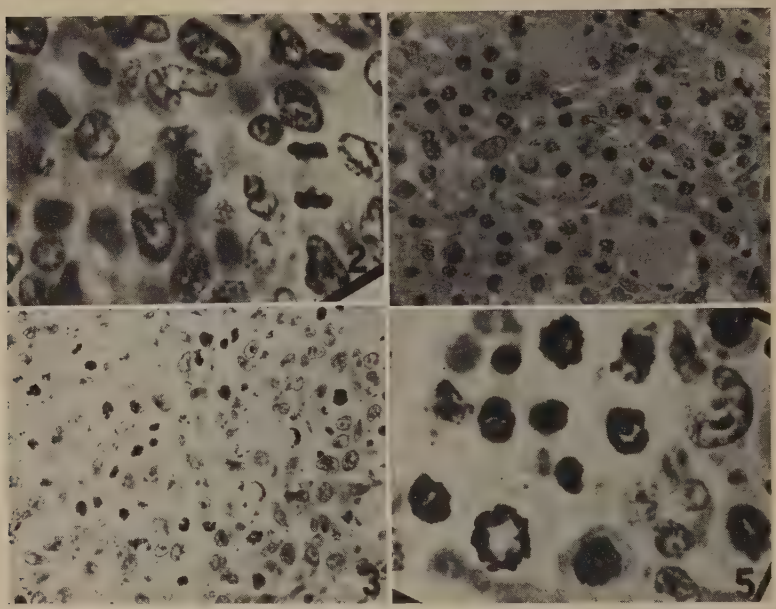

FIGURE 2. Control sarcoma 37. Zenker-formol. Haematoxylin/eosin. High power.
FIGURE 3. Sarcoma 37. Treated 24 hours with N-acetyliodocolchinol methyl ether *in vivo* by single dose of 300 micrograms per gram of body weight. Zenker-formol. Haematoxylin/eosin. Low power.
FIGURE 4. Same as FIGURE 3. Low power.
FIGURE 5. Same as FIGURE 4. High power.

indicates a change in the organic base with which the minerals may have been combined. It should be noted here that, in the cytoplasm of treated tumor cells subjected to high centrifugal force, an argentophilic component, the nature of which is unknown, is separated farther toward the centripetal pole than are argentophilic elements of control nontreated cells (FIGURE 8). These changes are being investigated further in view of Heilbrunn's (1928) finding that, in sea urchin eggs, the appearance of the spindle depends upon a preliminary gelation, and that suppression of gelation results in suppression of the spindle.

In healthy control sarcoma cells, the Golgi substance frequently appears as a small network on one side of the nucleus, but it appears as often as a focalized collection of small osmiophilic granules (FIGURE 9). In tumor cells

that are moribund after treatment with acetyliodocolchinol, the Golgi material appears first as a well-formed network (FIGURE 10) which later breaks up into small globules which become focalized in clusters resembling multi-

FIGURE 6. Control (*left*) and treated (Acetyliodocolchinol, *right*) cells of sarcoma 37. Microincinerated. Absolute-alcohol-formalin fixation. Cells in mitosis. White ash is the residue of calcium and/or magnesium.

FIGURE 7. Control (*left*) and treated (Acetyliodocolchinol, *right*) sarcoma 37 cells. Centrifuged in a Beams ultracentrifuge at 200,000 times gravity for ten minutes and then microincinerated.
Untreated Control Sarcoma-37 Ultracentrifuged Sarcoma-37
Ultracentrifuged DeFano-Silver After treatment with Acetyliodocolchinol

polar division centers around an arrested mass of metaphase chromosomes. The mitochondria in such treated cells simply become enspherulated, and in the late moribund stages few mitochondrial elements may be seen.

2. *Podophyllin.* Podophyllin, given in low doses (20 micrograms) over a short period of time, induces changes in sarcoma 37 tumor cells that are essentially identical to those of the methyl ether of acetyliodocolchinol. In

podophyllin-treated cells, observed in the living state from teased preparations of previously treated tumors, many clear vacuoles develop among the chromosomes arrested in metaphase. These seem to coalesce into a single

FIGURE 8. Control and treated sarcoma 37 cells. Resting and dividing cells. Centrifuged at 200,000 times gravity. Fixed by DaFano silver method. Broad bands of white granules (negative photograph) represent argentophilic material moved toward centripetal pole in treated cells.

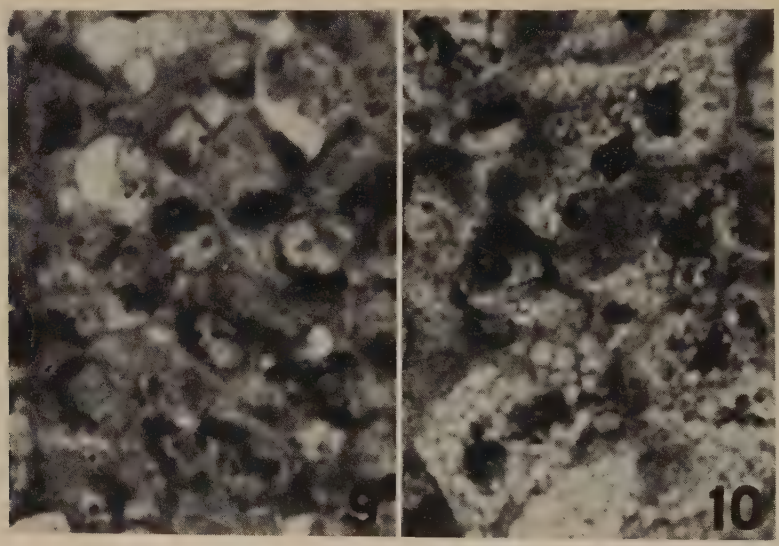

FIGURE 9. Control sarcoma 37 showing Golgi substance. Osmic acid.
FIGURE 10. Sarcoma 37 treated with N-acetyliodocolchinol methyl ether. Cells filled with intracytoplasmic globules. Golgi nets are black. Osmic acid. These cells are apparently moribund.

large sphere in the mid-plate region of the cell. A high dose of podophyllin induces stark necrosis at once, without preliminary distortion of mitotic figures. Necrosis without mitotic aberrations also can be induced by colchicine derivatives, anoxia, and anaesthetics. All of these changes observed in tumor cells after treatment with colchicine derivatives and podophyllin

may be seen in nontreated tumors around the periphery of an area of spontaneously induced necrosis in less widespread degree.

3. *Colchicine, Trypaflavine, and Arsenic.* The remarkable action of colchicine upon the mitosis of cells was first observed by Dustin (1933), who felt that this drug initially stimulated mitosis and then arrested movement of the chromosomes. It has been shown that administration of a low dose of colchicine temporarily arrests mitotic division of both normal and malignant cells in metaphase (Dustin, 1933; Lits, 1936; Ludford, 1937; Dalton, 1947). Colchicine suppresses spindle fiber formation and inhibits separation of the cell after the division of the chromosomes, so that the number of chromosomes is doubled. Upon removal of the drug, the nucleus divides in its normal fashion. Frog ova, fertilized with sperm previously exposed to colchicine, produce various anomalies (Hall, 1940). Colchicine arrests mitosis in metaphase in sarcoma 37. Other stages of division are rarely seen in arrest after treatment of sarcoma 37 with this drug. Colchicine produces effects similar to acetyliodocolchinol effects described above.

Trypaflavine prevents the onset of prophase and causes cellular degeneration but does not arrest mitosis (Dustin, 1925). According to Dustin and Piton (1929), arsenic is similar to colchicine in its action on mitosis.

It is interesting to note that P. Dustin, Jr. (1947) points out that mitotic figures inhibited by sodium arsenite proceed normally to telophase if treated in time with a dose of dimercaptopropanol (BAL). The effect of mitotic inhibitors on enzymes needs further research.

Summary

The most diverse agents can provoke similar alterations in mitosis. Their effects seem to be governed by the dose. At present, it is felt that none of the changes found in chemically treated sarcoma 37 cells is peculiar to any particular chemical agent studied. Chemical treatment induces translocation of iron from peripheral cytoplasm to midregion of tumor cells arrested in metaphase and accumulation of calcium and/or magnesium in the spindle area as revealed by microincineration.

Bibliography

Boveri, Th. 1901. Zellenstudien IV. Über die Natur der Centrosomen. Jena Zeit. f. Med. u. naturwiss. **25.**

Demoor, J. 1895. Contributions a l'etude de la physiologie de la cellule. Arch. de Biol. **13.**

Downing, V., J. L. Hartwell, J. Leiter, & M. J. Shear. 1949. Effect of a single injection of colchicine; colchicine derivatives and related compounds on mouse tumors. Cancer Research **9.**

Dustin, P., Jr. 1947. Some new aspects of mitotic poisoning. Nature **159:** 794.

Ephrussi, B. 1926. Sur les coefficients de temperature des differentes phases de la mitose des oefs d'oursin et de L'ascaris megallocephala. Protoplasma **1:** 105.

Fol, H. 1876. Sur les phenomenes de la division cellulaire. Comptes Rendu **83.**

Heilbrunn, L. V. 1928. Colloid Chemistry of Protoplasm. Borntraeger. Berlin.

Henshaw, P. S. 1940. Action of X rays on gametes of *Arbacia punctulata*. Amer. J. Roentg. **43.** 917.

Lea, D. E. 1947. Actions of radiations on living cells. MacMillan. New York.

Lits, F. J. 1936. Recherches sur les réactions et lésons cellulaires provoquées par la colchicine. Arch. Internat. de Med. exp. **11:** 811.

Ludford, R. J. 1937. Action of toxic substances upon the division of normal and malignant cells. Arch. f. Exper. Zellforchung. **18:** 411.

MacCardle, R. C. 1949. Changes in mitosis in sarcoma cells of mice treated with podophyllin and with colchicine derivatives. Cancer Research **9:** 555.
Mathews, A. P. 1907. A contribution to the chemistry of cell division, maturation and fertilization. Am. J. Physiol. **18:** 89.
Mitchell, J. S. 1942. Disturbance of nucleic acid metabolism by X and gamma rays. Brit. J. Exp. Path. **23:** 285.
Moulton, F. R. 1947. Approaches to tumor chemotherapy. Publication of Amer. Assoc. for the Adv. of Science.
Painter, T. S. 1916. Contributions to the study of cell mechanics. I. J. Exp. Zool. **23.**
Shear, M. J., V. Downing, J. L. Hartwell, J. Leiter, R. C. MacCardle, A. Perrault & D. L. Vivian. 1949. Preliminary screening of 1000 chemical agents for potency in producing damage in Sarcoma 37. Cancer Research **9.**
Sparrow, A. H. & F. M. Rosenfeld. 1946. X ray-induced depolymerization of thymonucleohistone and of sodium thymonucleate. Science **104:** 245.
Strangeways, T. S. P. & F. L. Hopwood. 1926. Effects of X rays on mitosis. Proc. Roy. Soc. B. **100:** 283.
Taylor, B., J. P. Greenstein, & A. Hollaender. 1948. Effects of X radiation on sodium thymus nucleate. Arch. Biochem. **16:** 19.
Wilson, E. B. 1901. Experimental studies in cytology. Arch. f. Entwicklungsmech. **12.**
Wilson, E. B. 1928. The cell in development and heredity. MacMillan. New York.
Wilson, J. W. & E. H. Leduc. 1948. The occurrence and formation of binucleate and multinucleate cells and polyploid nuclei in the mouse liver. Am. J. Anat. **82:** 353.

EFFECTS OF EXTERNAL AND INTERNAL RADIATION ON CELL DIVISION

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The effects of radiations on cell division have been extensively studied. Not only has it been found that ionizing radiations are a valuable tool in elucidating the mechanisms of mitosis, but much of our knowledge of quantitative radiobiology has come from studies of mitosis. Mitosis is one of the earliest functions to be affected by radiations, and, since radiation-induced death in higher organisms and isolated cells occurs after considerable delay, unless overwhelming doses are used, attention is naturally drawn to the earliest visible effects, among which the effects on mitosis occupy a prominent place.

The first effect is a suppression of mitosis. Those cells which are in mitosis at the time of radiation complete the cycle, while further division fails to occur. The duration and completeness of this effect are dependent on the amount of radiation given. Recovery from mitotic inhibition is associated with a compensatory increase in the proportion of dividing cells, in which a retardation of the mitotic cycle plays a part. It would appear that, over a wide range of moderate radiation dosages, tissue growth continues and only the process of mitosis itself is delayed. After recovery is completed, the same number of cell divisions has occurred as would have taken place in the absence of radiation.[1, 2]

Suppression of mitosis not only takes place in many tissues of the higher organisms, but has been observed and widely studied in tissue cultures and in various lower forms. An exception to the universality of this phenomenon has been pointed out by Bloom,[3] who has noted that, in various experimental animals, under conditions that make mitotic inhibition fairly general, the spermatocytes are unaffected.

The mechanism of the inhibition of mitosis by ionizing radiations is unknown. Mitchell,[4] in a series of studies made by ultraviolet microscopy, has found that radiation inhibits the conversion, in growing tissue, of cytoplasmic nucleic acid to nuclear nucleic acid. Since one of the more noticeable concomitants of cell division is the accumulation of desoxyribose nucleic acid in the chromosomes, apparently at the expense of cytoplasmic ribose nucleic acid, a specific inhibition of this process might explain in part the unique nature of post-radiation mitotic inhibition, which apparently occurs without disturbance of many cell functions. Since mitosis is prolonged, as well as delayed, following radiation, it is likely that physicochemical processes also play a part.

With recovery from mitotic inhibition, various other alterations in cell division occur which are manifested as changes in the configuration of the dividing cell. The primary, or "physiological," changes include defects in the orientation of the mitotic figure, anomalies in spiralization of chromosomes, and a phenomenon which has been called "stickiness," which, ac-

cording to a suggestion of Darlington,[5] may be due to accumulation of nonpolymerized nucleic acid around the chromosomes.

Structural alterations of another type have been extensively studied in various types of material. Fragmentation of chromosomes is presumed to occur as a result of breaks in the chromosome or chromatid due to local ionization. It is believed that the great majority of such breaks are healed without later microscopic evidence of damage, although, of course, loss of genic material might occur under these circumstances. Chromosome bridges, which are seen at anaphase or telophase, are generally due to abnormal healing of chromosome breaks. Such bridges may occur when two sister chromatids are broken near the same point and a single chromosomal element finds itself attached to both spindles. The free remnants are then seen as two equal and unattached chromosomal elements.

These structural alterations may be seen long after irradiation. Those incurred at the time of a cell division, however, may not be visible until the next mitosis. Death of a cell frequently occurs at mitosis from changes visible at the previous mitosis. The chromosome varies greatly in its sensitivity to induction of these changes by radiation, depending on the stage of the mitotic cycle in which the injury is sustained. Under specified conditions, the extent of fragmentation, bridge formation, or other structural changes is quantitatively related to the amount of radiation. It is thus possible to make a quantitative comparison of various types of ionizing radiation by counting the incidence of these changes. Where such intercomparisons have been made, it has been seen that the distribution and density of ionization are important factors controlling the probability of occurrences of structural changes.[6]

There seems to be little doubt that most, and quite likely all, of the changes induced by ionizing radiations are mediated through chemical effects of ionization. The ionization of water is best understood and is probably of the greatest importance. As a result of radiation, free H and OH radicals are formed. The lifetime of these radicals is extremely short and they disappear largely through recombination. A variety of other combinations occurs, however, including H_2O_2. These are mainly oxidants, and the probability of their occurrence and of recombination to water depends on many factors, including ionization density and the presence of acceptors for the various radicals.

Very little of the quantitative work on chromosome changes following exposure to ionizing radiations is due to studies on the normal organs of higher animals. Also, most of our knowledge in this field is based on relatively brief exposures to large dosages of ionizing radiation. Because of the current importance of absorbed radioactive isotopes, which may create conditions of continuous low-level irradiation of various organs, I wish to discuss preliminary results of experiments carried out with the use of isotopes.

The regenerating rat liver was used in these experiments. This organ, although normally in a nongrowing state, is capable of very rapid growth following subtotal removal in the adult animal. The growth rate is such that a doubling of mass occurs in about two days, with a mitotic index in

excess of two per cent at the height of cell division. The large size of the cell and its nuclear components make it very favorable for cytological study. In this organ, rapid growth takes place without much evidence of alteration in the functions of the differentiated cell.

The rate of cell division follows a fairly consistent pattern after operation, with mitosis beginning (in the young adult rat) about 24 hours later and gradually decreasing in rate as the organ is restored.[7] Variations in mitotic rate from hour to hour are very considerable, so that sections from different rats taken at a given time vary greatly in mitotic index, and a large series of such specimens must be taken to establish the rate of cell division during any period of time.[7] The distribution of mitoses throughout a single liver is strikingly uniform, however, and whatever may be the conditions

TABLE 1

MITOTIC INDEX (PER CENT OF CELLS IN MITOSIS) 48 HOURS AFTER PARTIAL HEPATECTOMY

Controls	*48 hours after single dosage of total-body X ray*			*Focal β radiation by yttrium^{91} bead (48-hour exposure)*	
Mitoses %	*Dosage r*	*Mitoses %*	*% abnormal figures**	*Estimated dosage rep/hour*	*Mitoses %*
2.9	10	0.7		0.1	1.46
1.2		1.5		0.3	1.44
0.9	30	1.1		0.8	0.68
0		4.9		2.1	0.09
1.2	100	1.8	6	25	0.01
2.8		2.7		90	0
	300	0.9	24		
		3.3			
mean 1.5	1000	<0.05			
σ 1.1	3000	0			
	controls (mean)	1.5	4		

* Figures showing bridges per hundred anaphases and telophases.

determining mitotic rate at any instant, they must be the same throughout the liver.

Various forms of radiation treatment have been applied 24 hours after partial hepatectomy, and they have been followed by examination of the tissue 48 hours later. These include total-body X radiation given in a single dosage (dosage rate, 20 r/minute), injection of Pu^{239} and P^{32}, and continuous exposure of a liver to a point source of β rays, using small glass beads[8] containing carrier-free Y^{91}. Untreated regenerating livers were also examined, and a few livers were allowed to regenerate a second time by performing a second subtotal hepatectomy two weeks after the first.

Because of the variation in the normal mitotic rate as a function of time and unknown factors, illustrated in TABLE 1, only the yttrium^{91} bead experiment appears to be of quantitative value in determining the degree of suppression of mitosis. The other experiments illustrated in TABLE 1 cover

a wide range of radiation dosages and the data suggest that recovery from a single dosage of X radiation has not begun 48 hours later, where 1000 and 3000 r were given. These are total-body dosages that would be lethal to the whole animal.

In using yttrium^{91} beads, those point sources were inserted deep into the residual livers by means of a pair of fine watchmaker's forceps at the time of partial hepatectomy, and the tissue was examined 48 hours later.

FIGURE 1. Mitotic map of regenerating liver showing decrease in frequency of mitosis with increase in dosage. X marks the position of yttrium^{91} bead.

A map was made of a section of liver passing through the point where the center of the bead lay, and mitotic indices were computed in areas defined by concentric rings. The radiation dosage was calculated on the basis of the physical characteristics of the Y^{91} beta ray and its absorption in tissue. This calculation is subject to a systematic error due to the growth of the

FIGURE 2 (*See facing page*). a. Anaphase with bridge and acentric fragments. Note similarity to rad iation effect. Untreated regenerating liver, hematoxylin-eosin. Magnification, 2500×. b. Double metaphase, one half with aberrant fragments. Untreated. Feulgen-fast green. Magnification, 2500×. c. Late metaphase with multiple bridges or lagging chromosomes. This is a fairly common picture. Untreated. Hematoxylin-eosin. Magnification, 2500×. d. Degenerating cell in untreated regenerating liver. Feulgen-fast green. Magnification, 2500×. e. Polyploid nucleus (probably 8 times normal volume) in untreated liver on second regeneration. Feulgen-fast green. Magnification, 2500×. f. Residual nucleic acid, perhaps in channel between plates. Untreated. Feulgen-fast green. Magnification, 2500×.

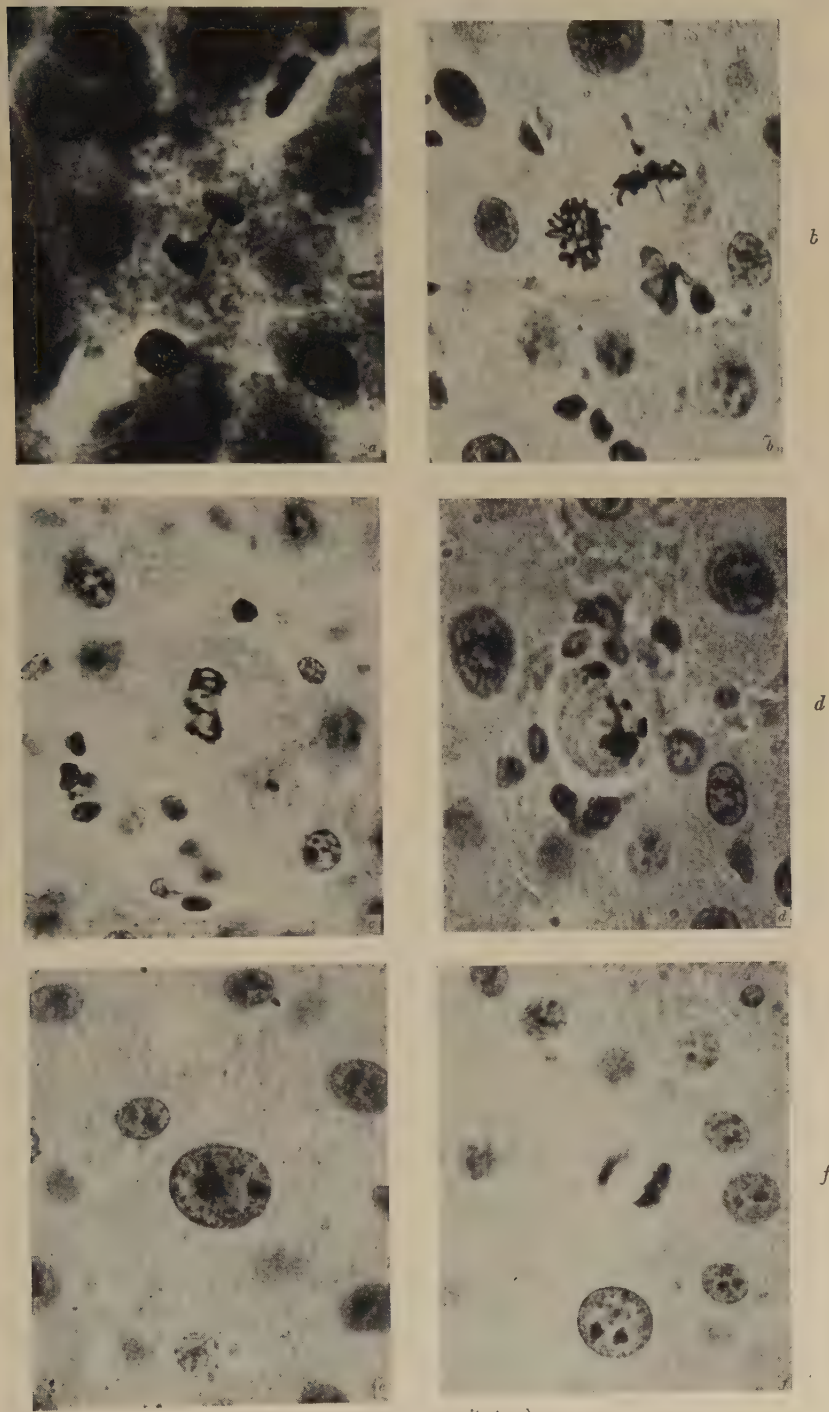

FIGURE 2. (*See opposite page*).

organ during the exposure period. This error, however, probably does not exceed 30 per cent in terms of tissue dosage at a given point. The dosage required to reduce the mitotic rate by one-half appears to be about one roentgen equivalent per hour over a 48-hour period (TABLE 1, FIGURE 1).

Examination of the fixed tissues, stained by hematoxylin or by the Feulgen method, has revealed a number of mitotic abnormalities characteristic of cytological radiation damage. These are illustrated in FIGURES 2–4.

It might be expected that the number of abnormal figures (*e.g.*, bridges and visible chromosome fragments) would be a sensitive index of the dosage of radiation used. Due to an unexpectedly large number of such figures in control material (TABLE 1), however, it would seem that significant increases above this "background" of abnormal mitoses can be found only when the radiation dosage is 50–100 r or higher. The frequency of abnorma figures above this background in irradiated material corresponds to rates observed in diverse materials.[9]

Since the background abnormalities were so striking, the finding has been verified in material collected by the author several years ago in a laboratory where no radioactivity was used, in rats killed by decapitation without anesthesia, and in young (50-gram) rats. In all cases, the value approached that shown in the table. Similar findings have been reported in the mouse.[10] The morphology of some of these abnormal figures is shown in FIGURE 2. Two instances of a bridge with acentric fragments (FIGURE 2, a) have been seen, and occasional degenerating cells of a type seen frequently in irradiated material are observed (FIGURE 2, d). It seems improbable that these phenomena can be attributed to cosmic radiation, although one might suppose that the liver may be subject to insults of a chemical nature, which could account for the phenomena.

A few livers, both irradiated and nonirradiated, were subjected to a second hepatectomy in order to see whether an increase in the rate of abnormalities could be detected in further mitoses. The only significant finding was an apparent increase in the number of polyploid cells.

Several types of abnormality are shown in the plates. It is of interest to observe the fate of chromosome bridges. In some cases, physiological changes appear to be concomitant with the appearance of the bridge, and a ring-like structure of the anaphase plate can be seen (FIGURE 3, a and b). In the latter case, the two halves of the figure may fail to separate and the bridge appears to have contracted (FIGURE 3, b). In other cases, a similar picture is seen without a visible bridge (FIGURE 3, c), as the bridge appears to have broken (FIGURE 3, d). Multiple bridges or lagging chromosomes in early anaphase (FIGURE 2, c) are a common finding in all of the material and may represent a normal mode of division in liver. The presence of a

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FIGURE 3 (*See facing page*). a. Chromosome bridge and large acentric fragment with fine structure. 30 r X ray. Feulgen-fast green. Magnification, 2500×. b. "Ring" anaphase with bridge which has apparently contracted. 30 r X ray. Feulgen-fast green. Magnification, 2500×. c. Similar picture; bridge not seen. Note appearance of clear areas in the ring structure suggesting early telophase picture. 300 r X ray. Feulgen-fast green. Magnification, 2500×. d. Early telophase with picture suggesting a broken bridge. 100 r X ray. Fuelgen-fast green. Magnification, 2500×. e. Multiple acentric fragments, one with filamentous attachment. Plutonium,^{239} 75 micrograms/rat. Hematoxylin-eosin. Magnification, 2200×. f. Multiple fragmentation with filaments, one partly coiled. 300 r X ray. Fuelgen-fast green. Magnification, 2500×

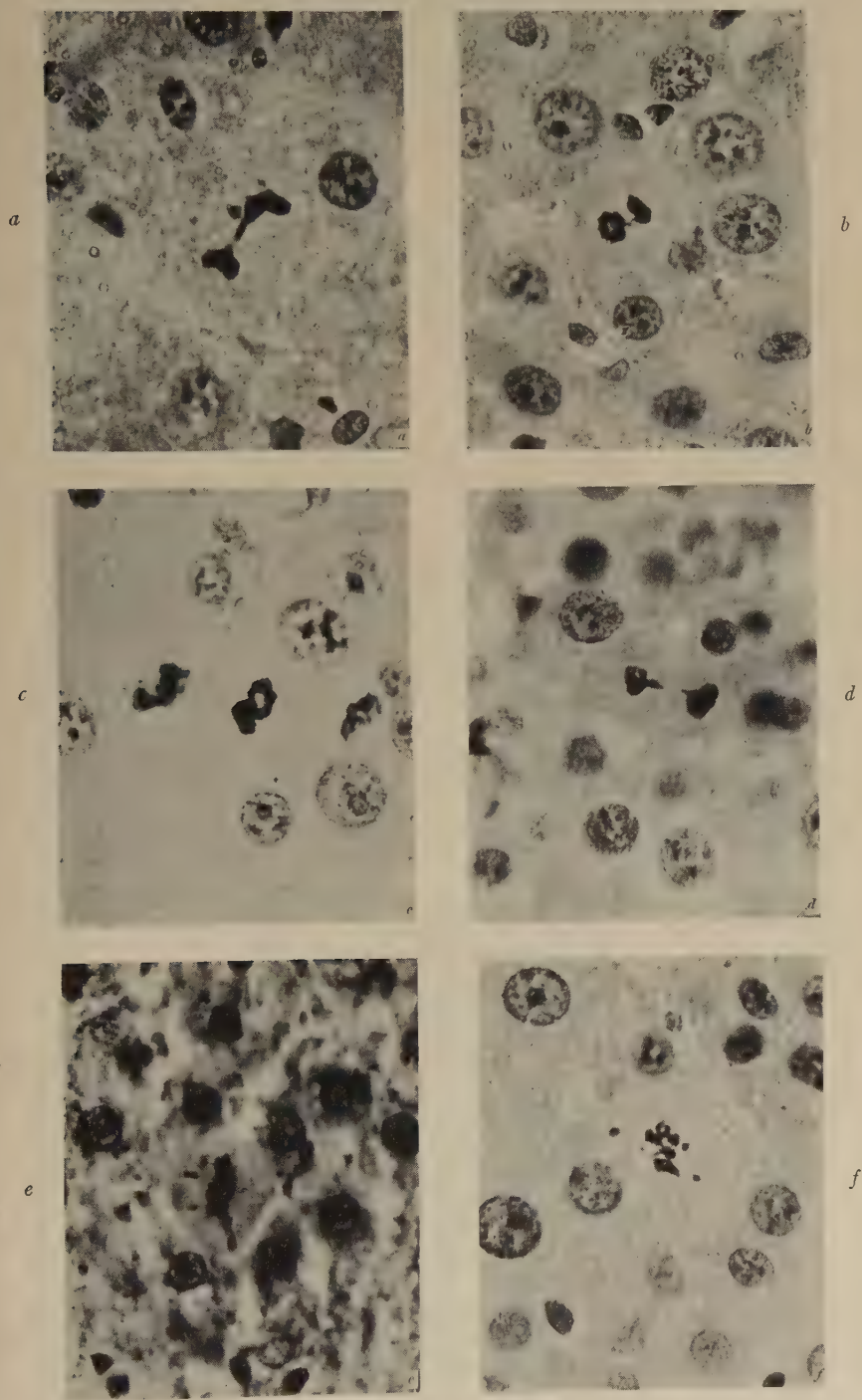


FIGURE 3. *(See opposite page).*

small amount of desoxyribose nucleic acid in a channel between the anaphase plates (FIGURE 2, f) may also occur independently of radiation. Of the various physiological changes noted, "stickiness" and depletion of nucleic acid, or failure of chromosomes to show the normal degree of mitotic coiling (FIGURE 3, e and f; FIGURE 4, a), are generally associated with irradiation damage.

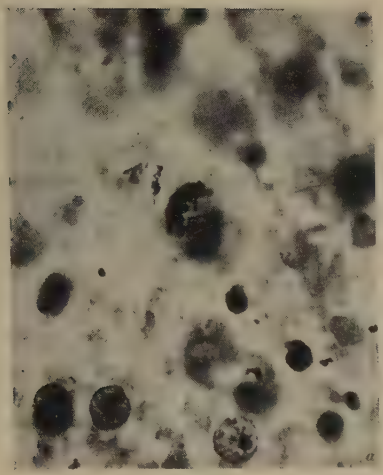
Much later effects of repeated or continuous irradiation are strikingly shown in livers of rats treated with plutonium over a long period of time and in tumors in the process of regression after repeated X-ray treatments. Here we see marked variation in nuclear size and structure, and extremely polyploid cells (FIGURE 4, b and e), with estimated nuclear volumes over 50 times normal. In liver, large vacuole-like structures are seen which may be swollen nucleoli (FIGURE 4, c). These cells are frequently seen degenerating, as indicated by shrinkage and loss of normal structure of the cytoplasm (FIGURE 4, d).

The question is often raised whether radiosensitivity of an organ is related to cell division and growth rate. It appears to be generally true that this is the case, since the blood-forming organs, the germinal cells, and the intestinal mucosa will show degenerative changes following amounts of radiation which leave most organs morphologically intact. Some exceptions exist, however. The mature lymphocyte is known to be highly sensitive to radiation,[3] while the regenerating liver has shown no evidence of increased sensitivity, although its growth and mitotic rate are at least as rapid as are seen in highly sensitive materials. Examination of nondividing cells of the same liver in regions of high and low dosage from Y^{91} β rays (FIGURE 5) fails to show visible differences, although the total dosage varies between 3 and 5000 rep. It seems possible that radiosensitivity may be related to a high ratio of nuclear to cytoplasmic nucleic acid, since the highest such ratio occurs in lymphoid tissue and the lowest ratios are in radiation-resistant cells.

It is also worth while considering to what extent overall damage from radiation may depend on the anomalies of mitosis resulting in the death of sensitive cells and caused by ionization in the region of the chromosomes. It is noteworthy that some isolated tissues in culture may withstand relatively enormous amounts of radiation, on the order of 50,000 roentgens, and the same is true of many simple organisms, while death in the higher animals occurs after dosages of a few hundred roentgens. This lower dosage range represents the region in which chromosome damage is widespread within a single cell.

The lethal effects of radiation in the higher animals are associated with destruction of radiosensitive blood-forming elements, and the failure of the blood-clotting mechanism has been related to an increase in heparinoid substances in the circulation, which may be released by certain radiated cells.[11] Ulceration of the sensitive intestinal epithelium may increase the probability of general infections, while resistance to bacterial invasion may

FIGURE 4 (*See facing page*). a. Aberrant chromosomes (possibly part of a second figure) showing partial coiling and "nucleic acid starvation." Stickiness of chromatin is also noted. Plutonium,^{239} 25 micrograms/rat. Hematoxylin-eosin. Magnification, 2500×. b, c, and d. Nuclei in liver (unregenerating) 60 days after in early regression following 2700 r X ray (300 r daily) Hematoxylin-eosin. Magnification, 1800×.



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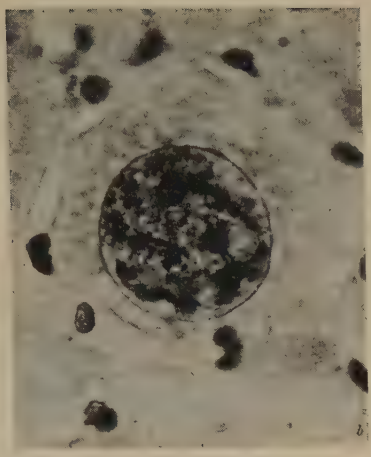
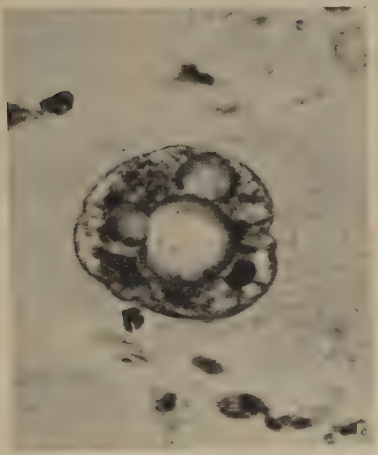
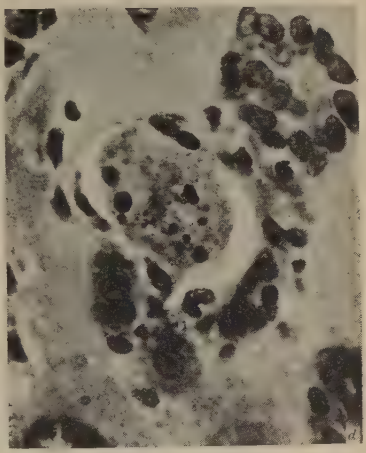


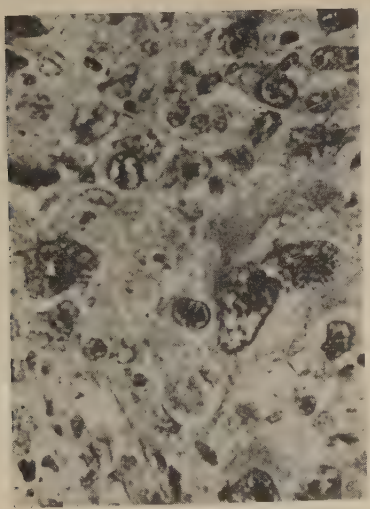
b



c



d



e

FIGURE 4. (*See opposite page*).

have been lowered through damage to the blood-forming tissues. It is also worth recalling that the radiomimetic chemicals, such as the nitrogen mustards, produce chromosome damage and a physiological state in the whole animal, both of which are closely similar to the effects of ionizing radiation. The latent period before radiation death, except where excessive dosages are applied, is sufficient to allow time for the consequences of death of the radiosensitive cells to become manifest on the physiological level.

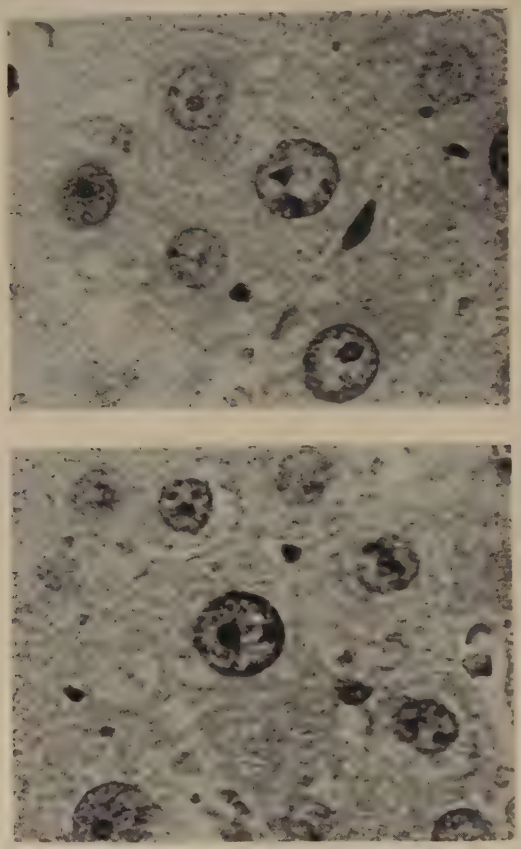


FIGURE 5. Comparison of nonmitotic cells in regenerating rat liver treated with local beta irradiation by Y^{91}. Top, area of minimal dose (not over 10 rep); bottom, area near bead (approximately 20,000 rep). Feulgen-fast green. Magnification, 1100×.

The possibility that the induction of tumors by ionizing radiations may be related to genic changes in cells may also be given serious consideration. In view of the widespread damage to chromosomes which follows dosages of radiation sufficient to induce tumors, it would seem not unlikely that the development of neoplasia might be attributable to the occurrence of a very rare, highly specific change, on the assumption that the tumor arose as the result of an irreversible nonlethal change in a single cell.

The regression of tumors under radiation therapy probably involves sev-

eral processes, including damage to the vascular bed and direct cell destruction. It is also possible that delayed death of irradiated tumor cells may be attributable to chromosome damage. The most remarkable nuclear changes are seen, not immediately after radiation, but when the tumor is regressing most actively several days later. These changes suggest late effects of chromosome injury becoming manifest in the descendants of irradiated cells.

Conclusion

The important effects of ionizing radiations on mitosis include reversible delay in cell division, followed by visible chromosome damage and "physiological" changes in the mitotic figure.

The nonirradiated regenerating rat liver has been found to contain many mitotic figures resembling radiation effects. Radiation by X rays or by internally absorbed radioelements increases the number of abnormal figures and inhibits mitosis. The amount of β radiation required to reduce liver mitosis by one-half is of the order of one roentgen equivalent per hour over a forty-eight-hour period.

Consideration has been given to the possibility that radiation death in the higher animals, and the induction and regression of tumors due to radiation, may all depend primarily upon chromosome damage.

Acknowledgement

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Bibliography

1. Spear, F. G. 1932. Effect of spaced radiation on tissue cultures *in vitro*. Proc. Royal Soc. B. **110:** 224.
2. Knowlton, N. P., Jr., L. H. Hempelmann, & J. G. Hoffman. 1948. The effects of X-rays on the mitotic activity of mouse epidermis. Science **107:** 625.
3. Bloom, W. 1947. Histologic changes following irradiation. Radiology **49:** 344.
4. Mitchell, J. S. 1943. Metabolic effects of therapeutic doses of X- and γ-radiations. Brit. J. Rad. **16:** 339.
5. Darlington, C. D. 1942. Chromosome chemistry and gene action. Nature **149:** 66.
6. Catcheside, D. G. 1947. Survey of effects of radiation on chromosomes. Brit. J. Rad. **1**(Supplement): 66.
7. Brues, A. M. & B. B. Marble. 1937. An analysis of mitosis in liver restoration. J. Exp. Med. **65:** 15.
8. Kisieleski, W., G. Svihla, & A. M. Brues. 1950. Preparation of radioactive glass-beads. Science 112: 400.
9. Lea, D. E. 1947. Actions of Radiation on Living Cells: 236. Cambridge University Press.
10. Wilson, J. W. 1948. Personal communication.
11. Allen, J. G., M. Sanderson, M. Milham, A. Kirschon, & L. O. Jacobson. 1948. Heparinemia (?) An anticoagulant in the blood of dogs with hemorrhagic tendency after total body exposure to Roentgen rays. J. Exp. Med. **87:** 71.

RADIATION SENSITIVITY OF CELLS DURING MITOTIC AND MEIOTIC CYCLES WITH EMPHASIS ON POSSIBLE CYTOCHEMICAL CHANGES*

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A number of recent reviews (Catcheside,[46, 47] Dobson and Lawrence,[85] Draver,[91] Fano and Demerec,[108] Giese,[122] Goodspeed and Uber,[130] Henshaw and Francis,[147] Hollaender,[154] Lavedan,[197] Lea,[201] Spear,[322] Timofeeff-Ressovsky and Zimmer,[345, 346] and various papers in Brit. Med. Bull. **4**: 1, and Suppl. 1, Brit. J. Radiol., 1946) are concerned mainly with the effects of radiations on living cells, but, for the most part, they do not give adequate consideration to the problem of sensitivity changes or to the interrelationships between radiosensitivity and the various factors which may cause an increase or decrease in cell sensitivity. In the present review, particular emphasis will be given to the changes in radiosensitivity which occur during the meiotic and mitotic nuclear (or chromosome) cycles, and an attempt will be made to summarize the existing information on the basis of physiological, biochemical, or cytochemical factors which may seem pertinent to the general problem.

Differences in the radiosensitivity of cells and tissues not only are of theoretical interest but are of considerable practical importance. Differences in susceptibility of neoplastic *vs.* normal tissue has long been utilized in X ray and radium therapy, but the practice is largely empirical, since the reasons for the difference are not well understood. This is not too surprising, however, since the fundamentals of the mechanism (or mechanisms) of the biological effect of radiations on living cells are likewise only partially understood.

The interaction of X-ray photons, or other ionizing particles, with protoplasm is generally considered to be mediated by excitations and/or ionizations, either directly or indirectly. This is, however, only the first step in a chain of events which ends in a recognizable biological effect. The intermediate steps are probably, in most cases, a complex series of events or reactions which in some instances, *e.g.*, mutations, result in a stable chemical change in certain protein or nucleoprotein constituents of the nucleus, and, in others, in unstable or degenerative changes. It is highly probable that most, if not all, species of organic molecules present in a cell suffer some change as a result of the energy transformations involved in the absorption of ionizing radiation. The effect on the biological functions of the cell, however, may not be proportional to the total number of all molecules affected but, rather, dependent upon the damage caused to particular molecular species or specific loci which are present within the cell in limited numbers and are involved in essential activities of cellular physiology or coordination.

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In view of the complexity of both cell structure and physiological processes, it is not surprising that the effects of radiation may be manifested in many different ways. As a result of this, a variety of methods has been devised for studying and measuring the effects of the radiation, whether they be immediate or delayed, temporary or permanent (*e.g.*, genetic), direct or indirect, physical or chemical, physiological or morphological, or a combination of two or several of the possible modes of expression.

Since many factors are known to affect the radiosensitivity of cells (see p. 1518) at any given time or stage of development, the ability of a cell or organism to resist or repair radiation damage must necessarily be dependent upon a number of external and/or internal environmental or cytochemical conditions or states. It is the author's intention to try to correlate such changes with radiation sensitivity at different stages of nuclear division. This will not be an easy task, since there is much disagreement, not only concerning relative radiosensitivity of various stages, but also concerning the many associated cytochemical or cytophysiological changes which can and do occur.

I. General Significance of Induced Chromosome Breakage

A large proportion of this paper will deal with the problems connected with chromosome breakage. The following points will serve to illustrate the main reasons why chromosome breakage and rearrangements are of importance in radiobiology:

(1) Chromosome breakage can be studied (at least in many cases) within a few hours after treatment. This affords a certain advantage over the genetic method, which may require a much longer time. (2) Breakages are known to be associated with radiation-induced mutations and, in many cases, to be responsible for lethal or semilethal mutations (see Herskowitz[148] for references). (3) Various types of permanent chromosomal aberrations (inversions, translocations, *etc.*), which may lead to partial or complete sterility, are induced. (4) Various types of induced aberrations have been valuable to the geneticist in certain problems, such as the location of particular genes in the *Drosophila* salivary gland chromosomes, and in the study of position effect. They may also contribute to our knowledge of mechanisms involved in crossing over and spiralization. (5) Chromosome breakage appears to be responsible for mitotic inhibition directly, and also indirectly, through loss of chromatin by fragmentation. These two effects probably play a significant part in the effect of radiations on growth in both normal and malignant tissue. (6) Fragment counts on biopsy specimens have been used successfully to determine effectiveness of irradiation in human tumors and as a basis for adjustment of further radiation treatment.[187] (7) If we accept the theory that somatic mutation can produce cancer (as suggested by Lederberg,[204] and others, references in Loeb[213]), chromosome breakage or mutation associated with breakage would be responsible for a certain percentage of radiation-induced malignancies.

The biological effect of ultraviolet or ionizing radiations may be studied by observations on one or more of the following: (1) chromosome fragmen-

tation (terminal and interstitial deletions, isodiametric or rod-shaped fragments, acentrics, *etc.*); (2) chromosome aberrations resulting from recombination of broken ends (translocations, inversions, insertions, repeats, bridges, rings, polycentrics, sister chromatid rejoins, *etc.*); (3) abnormal chromosome spiralization; (4) other cytological or histological effects (chromosome clumping, atrophy, *etc.*); (5) mitotic inhibition (or mitotic index); (6) other cytochemical or histochemical changes; (7) mutation rate (of lethal, visible, or biochemical mutants); (8) sterility or semisterility; (9) percentage of deaths or percentage surviving; (10) growth (in size, length, or weight) or a lack of it, or regression; (11) various physiological reactions, including enzyme or hormone inactivation; and (12) physico-chemical effects, such as change in viscosity, permeability, protoplasmic streaming, *etc.*

A number of those listed above have been used to study relative radiosensitivity of different stages of meiosis and mitosis. The results of a considerable number of investigations are summarized in TABLE 1. Almost every stage has been claimed to be the most (or least) sensitive. This could be ascribed in some cases to the different experimental organisms used, but it seems highly doubtful that this factor alone can account for all the discrepancies. These will be discussed later, after considering the sensitivity curve obtained by the author for meiotic stages of *Trillium erectum*.

II. Sensitivity Changes in Trillium

Trillium erectum offers a number of advantages for studying relative sensitivity of different stages of the nuclear cycle[321a] (see FIGURE 1). Meiotic divisions are generally well synchronized, and the anthers contain a large number of microsporocytes. It is thus possible to irradiate many thousands of cells at any desired stage and to study the effect on subsequent meiotic divisions, or on either of two post-meiotic mitotic divisions. Each of these four divisions can be distinguished readily, so that it is possible to know whether observations are being made on the first, second, or third divisions following irradiation. This is important because it is sometimes necessary, after irradiation at prophase, to count fragments at two or three succeeding metaphases (or anaphases). Failure to do this may give only a partial picture of the total fragmentation induced. Few, if any, of the previous investigators have used this double scoring method and, therefore, their data, in many cases, do not represent total sensitivity, but only a portion of the total effect.

The number of fragments observed at meiotic first anaphase or microspore metaphase, after irradiation with 50 r at various stages, is given in TABLE 2 and illustrated graphically in FIGURE 2. After irradiation at leptotene, zygotene, and pachytene, scoring was done at first anaphase. For later stages of prophase, two sets of data (one from AI and one from microspore metaphase) must be combined. The dotted line in FIGURE 2 illustrates the "sensitivity curve" one would obtain by scoring only at AI and accounts for reports by previous investigators that sensitivity decreases as metaphase is approached. If we also consider those breaks which develop

later (after the chromosomes have passed through an interphase*), however, a new sensitivity curve for late prophase stages is obtained, and it can be

TABLE 1

STAGES OF HIGH AND LOW SENSITIVITIES TO RADIATION AS REPORTED BY VARIOUS INVESTIGATORS

Organism	*Stage(s) of high sensitivity*	*Stage(s) of low sensitivity*	*Sensitivity change*	*Reference*
Chick	resting or very early prophase	metaphase & anaphase		Strangeways & Hopwood (1926)
Chorthippus	very late interphase			Creighton (1941)
Chortophaga	prophase (mid)			Carlson (1942)
*Gasteria**	pachytene		15 ×	Marshak (1935, 1939)
*Vicia**	pachytene		2 ×	Marshak (1938b)
Tradescantia	prophase			Sax & Swanson (1941)
Tradescantia	decreases toward metaphase			Sax (1940)
Psammechinus	prophase & telophase	metaphase		Langendorf & Langendorf (1931)
Rana (Ra)	prophase & telophase	metaphase		Luther (1938)
	anaphase & prophase			Regaud (1923)
Allium	metaphase	interphase	37 ×	Conger (1947)
Tradescantia & other genera	increase toward metaphase			Newcombe (1942)
Tradescantia	probably metaphase or anaphase		10 ×	Bishop (1950)
*Trillium**	late prophase — metaphase	early interphase	50 ×	Sparrow (1948)
Ascaris (Ra)	metaphase	resting	8 ×	Mottram (1913)
*Ascaris**	metaphase & anaphase			Holthusen (1921)
Arbacia (Ra)	metaphase	interphase		Packard (1916)
*Habrobracon**	metaphase I	late prophase	32 ×	Whiting (1940, 1945a)
Sciara	metaphase & anaphase	prophase		Bozeman (1943), Reynolds (1941)
Rana	anaphase & telophase	resting	6 ×	Vintemberger (1928a)
Mouse	interphase, then telophase			Marshak (1942)
Allium		metaphase		Marshak (1938 a, b)
Tradescantia		late resting to early prophase		Koller (1946)

(Divisions mostly mitotic. Those marked * were meiotic. Radiation was X rays except those marked Ra (radium)).

seen that sensitivity continues to increase after pachytene. Diplotene, diakinesis, and first metaphase (FIGURE 1) are all stages of high sensitivity

* There is normally no regular interphase between first and second meiotic divisions in *Trillium erectum*. The chromosomes remain condensed and pass directly into second division.

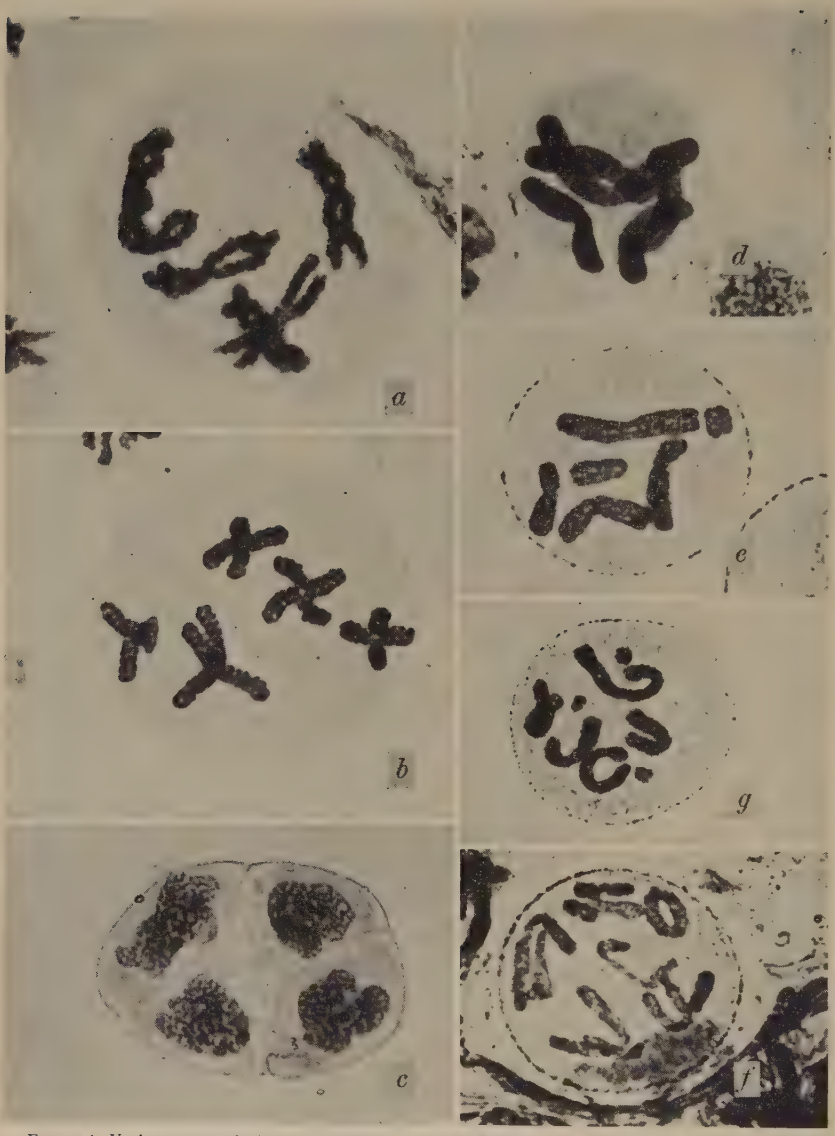

FIGURE 1. Various stages of microsporogenesis in *Trillium erectum*. Photo-micrographs were made from propiono-carmine permanent smears. Magnification approximately 750×. (a) Late diplotene or early diakinesis. (b) First metaphase showing five pairs of bivalents (chiasma frequency unusually low). (c) Early interphase. (d) Normal microspore metaphase. (e) Microspore metaphase showing one fragment. (f) Microspore metaphase showing three fragments. (g) Microspore anaphase showing a dicentric bridge (near middle of cell) and ring shaped chromosome (top right).

(more data are needed for diplotene and diakinesis before it can be decided which state is the more sensitive). After first metaphase, sensitivity decreases steadily throughout AI and second division and reaches a low point in early (seven-day) post-meiotic interphase (FIGURE 1 c). The curve then rises during later interphase stages. Data are now being collected to deter-

TABLE 2

RELATIVE SENSITIVITY OF *Trillium* CHROMOSOMES TO X-RAY BREAKAGE AT VARIOUS STAGES OF MICROSPOROGENESIS (DOSAGE IN ALL CASES WAS 50 r)

Stage(s) irradiated	*No. of buds X-rayed**		*No. of cells scored**		*Fragments induced per 100 PMC**			*Average† relative sensitivity*
	For PMC scoring	*For MSP scoring*	*PMC*	*MSP*	*Scored at anaphase I*	*Scored at microspore metaphase*	*Total fragments*	
Leptotene-zygotene	7		2050		117.7			10.5
Pachytene	12	6	2211	577	124.8	76.7	201.5	18.0
Diplotene—early	9		2114		106.8			
—late	7		1399		40.5			
—average	16		3513		77.8	606.5	684.3	61.1
Late diplotene-diakinesis	4		1000		27.0			
Diakinesis-metaphase I	8	5	1377	346	13.1	551.1	564.2	50.4
Metaphase I	3	7	352	2300	6.8	556.3	563.1	50.3
Anaphase I		14		2630		366.8	366.8	32.8
Metaphase II—anaphase II		5		2027		194.4	194.4	17.4
Telophase II—very early interphase		4		1678		39.5	39.5	3.5
Interphase (7 day)		4		735		11.2	11.2	1.0
Interphase (21 day)		6		1001		271.3	271.3	24.2
Total.....	50	51	14,016	11,294				

* PMC = pollen-mother-cell; MSP = microspores.

† Calculated by assigning the least sensitive stage (7-day interphase) an arbitrary value of 1.

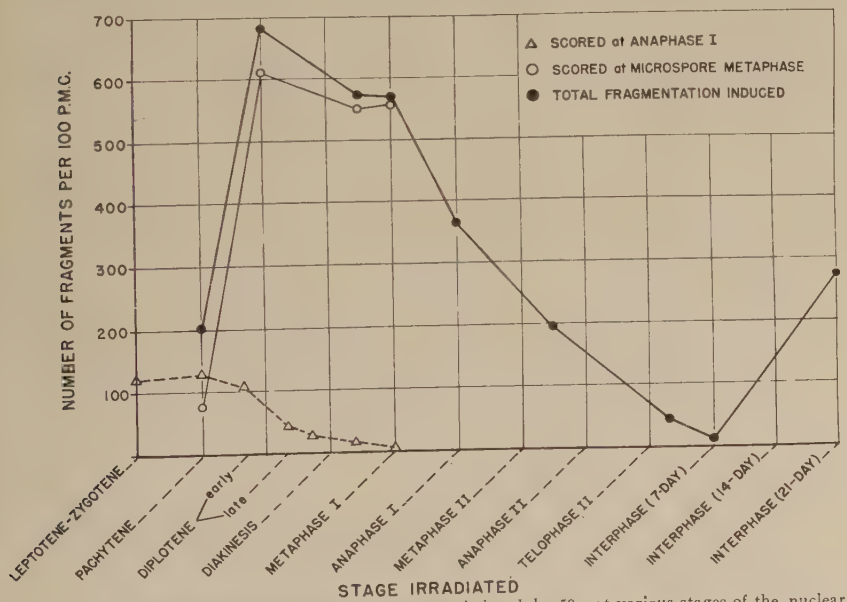

FIGURE 2. Graph showing the numbers of fragments induced by 50 r at various stages of the nuclear cycle during microsporogenesis in *Trillium erectum*.

mine what changes occur during late interphase, microspore prophase, and metaphase.

By assigning the least sensitive stage (early interphase) an arbitrary value of 1, it is possible to calculate the sensitivity of other stages relative to early interphase. The average relative sensitivities of all stages so far investigated are given in the last column of TABLE 2. The break frequency of the most sensitive stage(s) is about 50 times greater than that of the least sensitive. This is considered the minimum. It is expected that eventually an even greater difference will be demonstrated when more complete data are available. This change of sensitivity with the stage of division is somewhat greater than any previously reported (see TABLE 1) for either meiosis or mitosis.

It is the opinion of the author that more work on a variety of different organisms would confirm the claims that metaphase is a stage of high sensitivity. However, the exact stage of highest sensitivity need not necessarily always be metaphase. In other organisms, it might occur in late prophase or anaphase. This would not be surprising, as the physiological state and structure of the chromosomes in these three stages are probably more similar to each other than to other stages (*e.g.*, telophase or interphase). For the remainder of this paper, the condensed state of the chromosome (late prophase, metaphase, and early anaphase) will be considered to be a stage of high potential breakability* by X rays (or other ionizing radiations), and the early interphase (uncondensed or diffuse) chromosome will be considered a stage of low breakability. Exceptions will be noted and explanations of such instances will be attempted on the grounds of either inadequate technique or unusual chromosomal structure or behavior.

III. Distribution of Chromosome Breaks

It is generally assumed that radiation-induced breaks may occur in any part of any chromosome, and, one might, therefore, expect break loci to be distributed in a purely random fashion. Actually, however, there seem to be a number of cases of non-random breakage. These are summarized in TABLE 3. Most of these are special cases, such as heterochromatic regions, ring or acentric chromosomes, *etc.* It seems highly probable that the various euchromatic regions of normal chromosomes would show complete randomness in break loci. Such is the case for *Drosophila* chromosome breaks recovered in F_1 larvae after irradiation of mature males (Kaufmann,[169] Bauer *et al.*[16]). However, there are a number of cases in which there is an excess of two-break aberrations in which both breaks occur in one chromosome (references in Catcheside[47]). In *Tradescantia*, initial breaks are considered by Sax[289] to be distributed at random, but secondary factors affecting restitution cause an excess of both one-break and two-break aberrations at the proximal ends of the chromosome arms. Catcheside (p. 278)[47] considers that the results of various investigators "provide no evidence of any polarization in the chromosome such as would prevent random joining of broken

* The ionizations cause no immediate visible effect (except chromosome clumping or stickiness) but produce only "potential breaks" which develop into visible breaks later, *i.e.*, after an intervening interphase.

ends." However, the differential uncoiling found in heterobrachial chromosomes (Sparrow[316]) might interfere with random rejoining.

Breaks within the centromere can be induced by irradiation, but the frequency of such breaks must be very low or, alternatively, one portion of the divided centromere must become nonfunctional, since the number of centromeres does not normally increase following irradiation. Interference with centromere (or spindle) activity by irradiation is indicated by increased non-disjunction following X-ray treatment[288, 6, 232] and occasionally by polyploidy[317] and the formation of diplo-chromosomes.[38]

TABLE 3

SUMMARY OF REPORTED CASES OF NON-RANDOM BREAKAGE OF CHROMOSOMES BY X RAYS

Organism	*Region of high sensitivity*	*Region of low sensitivity*	*Reference*
Aloë	specific regions (two per long arm)	remainder	Camara (1940)
Bellevalia	distal ends	remainder	Marquardt (1942)
Crepis	proximal end of chromosome	remainder	Lewitsky & Sizova (1935)
Tradescantia (microspores)	proximal end of arm	remainder	Sax & Mather (1939)
Tradescantia (pollen tubes)	medial & distal	remainder	Swanson (1942)
Tradescantia	centric chromosomes	acentric fragments	Sax (1942)
Trillium	euchromatin	negative heterochromatin	Darlington & La Cour (1945)
Pisum	proximal ends	distal ends	Krajevoy & Rassuly (1935)
Circotettix	proximal end of arm	remainder	Helwig (1933)
Drosophila	various heterochromatic regions	euchromatin	Kaufmann (1939), Hinton (1941), Muller (1941)
Drosophila	right arm of third chromosome		Kaufmann (1943a)
Drosophila	various regions		Kaufmann (1946b)
Locusta	euchromatin	negative heterochromatin	White (1935)

IV. Breakage, Reunion, and Mutation

In most organisms, broken ends of chromosomes (or chromatids and possibly half chromatids) apparently can reunite with another broken end (at least in a certain percentage of cases). Bauer and LeCalvez,[17] however, claim that the broken chromosomes of *Ascaris* eggs show no tendency to rejoin, and, in *Drosophila*[333] and *Zea*,[236] broken ends behave like normal ends in some cases. If reunion occurs, it may restore the original gene sequence and be undetectable either genetically or cytologically, or it may reform in a new combination which is detectable cytologically or genetically or, in some cases, by both methods. A gene mutation may or may not be associated with a break locus.

Any change in the observed number of visible breaks or aberrations induced by a unit dosage (*i.e.*, a change in sensitivity) may be attributed to

(1) an initial difference in the number of breaks or potential breaks induced, (2) a change in the number of breaks which reunite (in either the original or a new gene sequence) or of potential breaks which fail to develop, or (3) a combination of (1) and (2). Newcombe[262] concludes "that irradiation not only breaks the chromosomes but also inhibits reunion of broken ends. This inhibiting effect increases with increasing dose, is greater when a particular dose is administered more rapidly, and affects both restitution and new reunion."

It is generally assumed that a high proportion of breaks primarily produced in chromosomes reunite in such a way as to be cytologically undetectable. The percentage of initial breaks which would survive as open breaks, or take part in structural rearrangements, has been calculated by Muller,[258] Newcombe,[261] Catcheside and Lea,[49] Lea,[201] Haldane and Lea,[139] Kotval and Gray[190] and others. Estimates of the proportion of visible and lethal mutations associated with gross chromosomal aberration indicate that both types of mutation tend to be associated with chromosome breakage.[84, 49, 203, 107, 323]

TABLE 4

COMPARISON OF RING AND BRIDGE FREQUENCIES INDUCED AT METAPHASE AND AT INTERPHASE STAGE, WHERE MICROSPORE ANAPHASE FRAGMENT COUNTS ARE APPROXIMATELY EQUAL

Stage	*Anaphase fragments* per 100 PMC*	*Percentage bridges*	*Percentage rings*	*Percentage bridges & rings*
Metaphase	566 ± 40.21	4.7	1.32	5.98
Interphase	577 ± 21.7	17.8	4.90	22.72

* Scored at microspore anaphase.

In order to find out whether differential rejoining might be a factor in influencing apparent changes in sensitivity, representative types of aberrations involving cytologically detectable rejoins have been studied in *Trillium*.[320] The percentage of bridges and rings induced following radiation at first meiotic metaphase (stage of high sensitivity) and early interphase (stage of low sensitivity) is given in TABLE 4. Using the number of fragments as an index of sensitivity, and the number of bridges and rings as an index of rejoining of broken ends, it is possible to determine the relationship between rejoining and chromosome radiosensitivity. The data (TABLE 4) indicate that differential rejoining does occur and that it probably plays a role in determining the apparent relative sensitivity of chromosomes to X-ray breakage. This factor alone, however, can account for only a small proportion of the observed differences in sensitivity between the most sensitive and least sensitive stages. The reason for the differential behavior of chromosome ends following radiation at different stages of the nuclear cycle is not understood (see discussion in section V). Newcombe[261] and Catcheside and Lea[48] suggest that spatial considerations may be an important factor influencing reunion. Kaufmann[173] has attributed an excess of

reunions (inversions of certain lengths) to a pattern of coiling which would increase the opportunities for reunion at distances equal to a single gyre of the mitotic spiral.

The above observations support somewhat similar conclusions reached by Newcombe.[261] He found breaks to increase late in the nuclear cycle (*i.e.*, approaching metaphase). A smaller percentage of the total breaks available appeared to be involved in both restitution and new reunion. He suggested a reduced speed of reunion late in the cycle as the factor inhibiting rejoining.

The inverse relationship between breakage sensitivity and the tendency of broken ends to rejoin may be of significance in connection with the study of mutation rate and of the frequency of certain types of aberrations. It is possible that the capacity or tendency toward rejoining is automatically reduced in those cells in which a large number of chromosome breaks occur in a single nucleus. This would account for the observed decrease in "two-hit" aberrations at higher dosages,[290] as well as reduced percentage of bridges and rings observed (see above) at the most sensitive stages in *Trillium*.

In the presence of a low frequency of rejoining, an increased percentage of acentric fragments or deletions would be expected. Thus, a higher proportion of lethality would occur. Paradoxically, therefore, if one were scoring for aberrations in the F_1 generation, following radiation of one or both of the parental gonads, the recovered aberrations would not necessarily represent a true picture of total chromosome breakage, since cells in the most sensitive stages would be the least likely to produce viable F_1's. This point should further emphasize the necessity of specifying the type, or types, of chromosome aberrations upon which a sensitivity curve is based. It is possible that a sensitivity curve based solely on recoverable rings, translocations, and inversions would differ radically from a sensitivity curve based on terminal and interstitial deletions. The recent work of Kotval and Gray,[190] showing a linear relationship between aberrations and dose, suggests that the sensitivity curve for alpha-radiation might be different than that for gamma ray, X ray, or neutron treatment.

Various estimates have been made of the relationship between chromosome breakage and mutation,[18, 121, 49, 148] and breakage and dominant lethals.[200] It is obvious that any such relationship will be valid only when a relatively constant number of breaks is induced (*i.e.*, when the majority of cells irradiated are in the same stage of sensitivity). Evidence of nonrandom occurrences of mutations in specific chromosomes or chromosomal regions (references in Spencer and Stern[323]) indicates that mutagenic sensitivity[183] as well as sensitivity to breakage (see section III) can vary within a chromosome. This is not surprising, as there are a number of physiological factors known to affect mutation rate. A number of other factors have also been shown to affect mutation: Lamy;[194] Auerbach, Robson, and Carr;[13] D'Amato and Gustafsson;[76] see also section V, parts 2 and 3, this paper.

If one is concerned mainly with the lethal effect of radiation, a sensitivity curve based on the number of terminal and interstitial deletions would probably be a more reliable indication of expected mortality than a sensitiv-

ity curve based on aberrations resulting from rejoins, since these, on the whole, would be much less lethal than the loss of a portion of the genetic material. This has been stated more briefly by Whiting:[361] "Fragmentation of chromosomes is not lethal. Loss of fragments is."

Probably the most reliable method of determining the sensitivity would be to determine what dosage given at the various stages of the nuclear cycle would be required to induce a constant percentage of cell lethals. Since this would be an exceedingly difficult task, the author has begun an experiment to construct a sensitivity curve in *Trillium* based upon the different dosages required to give a relatively constant amount of fragmentation for each stage irradiated. Preliminary observations indicate that such a sensitivity curve will not be in complete accord with one based on constant dosage and a variable number of induced fragments.

V. Factors Affecting Nuclear Radiosensitivity

The rhythmic changes in the physical appearance of the nucleus and chromosomes at different stages of mitosis and meiosis represent a structural, physiological, and chemical differentiation. In recent years, the structural changes have been recognized as cycles of spiralization and despiralization (or coiling and uncoiling) of the constituent chromonemata. This, in turn, seems to be correlated with a cycle of nucleic acid attachment to a basic protein framework. Interphase and uncoiled or loosely coiled prophase stages are considered to be low in desoxyribose nucleic acid, while the more condensed, tightly "packed," or coiled stages contain relatively more nucleic acid. These differences show up cytologically as differences in reaction to standard chromatin stains or in ultraviolet absorption and probably are of great significance in determining radiosensitivity of the various stages. These and other factors, both of the external and internal environment, will be considered below in regard to their known or probable effect on chromosome breakage, gene mutation, or general radiosensitivity.

1. *Morphological and Structural Differentiation Within the Nucleus.* Apart from the gross effect of division stages already discussed, a considerable number of structural or morphological changes occur during the mitotic and meiotic cycle which can affect radiosensitivity. Chromosome size, length of chromonemata, stage of spiralization, polyteny, polysomaty, presence of knobs (*e.g.*, in *Zea*) or of nuclear organizing regions or other heterochromatin (see TABLE 3), and matrix[242] or nuclear membrane, are all factors which would be expected to influence sensitivity. Chromosome size varies widely, not only in different genera, species, *etc.*, but also at different mitotic stages and, in some cases, even between the metaphase and anaphase (Sparrow,[314] Conklin[62]).

An exceptionally high resistance of the protozoan, *Holomastigotoides*, has been reported (Cleveland and Day [unpubl.], quoted in Sax and Swanson[297]). The chromosomes are large and would be expected to have a fairly high sensitivity. However, no aberrations were found with dosages up to 20,000 r. The chromosomes are highly chromatic at all stages. This fact, together with the characteristic persistent nuclear membrane, may be

important clues as to why fragmentation was not observed, since irradiated metaphase chromosomes (highly chromatic) usually show no fragmentation until an interphase has occurred and the nuclear membrane breaks down. It has been shown that certain Protozoa contain little or no thymonucleic acid at certain stages of division.[51, 239] This may be related to their high radioresistance (see also section V, part 2).

An increase in chromosome size, number of chromonemata, and amount of heterochromatin has been found in certain neoplastic tissue.[24, 21, 22] If changes in cross-section area of chromonemata also occur, we would expect this to have an effect on sensitivity, since Catcheside and Lea[48] consider that the ionization track of an electron, if shorter than the chromatid diameter, is relatively inefficient. They assume that only the densely ionizing tails of the electron tracks are effective in causing chromosome breakage.

Sensitivity might also be affected by atypical stages such as lamp brush chromosomes, a diffuse diplotene, as in *Habrobracon* (Whiting[362]), induced aberrations such as rings or acentric fragments, the size and shape of the nucleus or relative proximity of different chromosomes, the degree of polyploidy,[363, 120, 259, 310, 311, 227] or the presence or absence of extra whole chromosomes, accessory fragments, centrioles,[111] or of extra B- or Y-chromosomes. Extra Y-chromosomes appear to have an effect on other chromosomes in the nucleus, as does translocated heterochromatin upon adjacent regions[303] and extra chromatin (in the form of heterochromatic B's) upon nucleic acid metabolism.[82] It has been suggested that euchromatin and heterochromatin react differently to irradiation, mutation occurring in the euchromatin and the retardation of cell divisions being due to an effect upon the heterochromatin (Hollaender and Emmons[155]).

A relationship between radiosensitivity and the state of chromonematic contraction seems plausible. Measurements of chromonemata lengths in *Trillium* showed, at diplotene and diakinesis, that the chromonemata are very condensed and that their length is greater at both earlier and later stages of meiosis (Sparrow, Huskins, and Wilson[319]). The data reported above suggest that a rough inverse correlation exists between chromonemata length and susceptibility to X-ray fragmentation in *Trillium*. Manton[218] has found a contraction ratio of 4:1 in *Osmunda* and points out that "this order of difference is exactly comparable to the changes of length accompanying changes of physical state in the fibrous proteins." A lack of correlation between X-ray inhibition and total chromosome lengths in different cereals has been reported by Marshak and Bradley.[227] More measurements on other material are needed before general conclusions can be safely drawn.

Chromosome movement within the nucleus, due to stress of spiralization or to the force exerted during anaphase, has been suggested as a possible factor (Darlington,[78] [p. 558], Sax,[288, 292] Giles,[123] Swanson,[336] and Conger[60]). Goodspeed[129] and Whiting[362] have suggested that tension may also be important. The latter author, who found metaphase I to be a stage of high sensitivity in *Habrobracon*, suggested "that the degree of tension is due to conditions peculiar to the first meiotic division." A preponderance of breaks in the proximal region of chromosome arms has been attributed by

Sax and Mather[296] to additional torsional stress present close to the centromere. Sax found a lower break frequency in acentric chromosome fragments than in centric chromosomes.[292] He states that "initial breaks must be at random, but those which are involved in chromosomal aberrations are in some way controlled by the centromere so that more illegitimate fusions occur in the centric than in the acentric chromosomes. This effect of the centromere could be attributed to polarity and spatial relations in the case of unions between chromatids of different chromosomes, but the simple deletions involving only sister chromatids at a given locus also appear to be associated with mechanical stress which is related to the centromere." An excess of breaks, however, near the distal ends, as reported by Camara[37] and Marquardt,[220] cannot be explained on this basis. A study of distribution of induced breakage in chromosomes having a diffuse centromere should be of considerable interest.

If the loss of a centromere decreases sensitivity, it might be expected that extra centromeres might increase sensitivity (*cf.* Marshak and Bradley[227]). Apparently, this has not yet been tested, but Bauer[15] has reported that mutation is greater in ring X- than in normal X-chromosomes of *Drosophila*. Assuming that mutation rate is related to break frequency, Bauer's results might be explained on the basis of a greater stress present in the ring chromosomes during certain stages of coiling or uncoiling.

Although the nature of the forces which cause chromosomes to repel or attract each other is not understood (*cf.* Stern[328]), it is quite plausible that differences in these forces may bear some relationship to sensitivity. This seems especially probable because of the fact that the chromosomes have a high sensitivity during their repulsive phases of diplotene and diakinesis (these are also stages of terminalization in some organisms) and, conversely, the highly attractive phase characteristic of telophase and early interphase are stages of low sensitivity and relatively high rejoining (zygotene is also a stage of low sensitivity but not nearly so low as telophase or early interphase. See TABLE 2).[320]

Differences in the reactions of broken ends of chromosomes at different stages of meiosis and mitosis have been noted by McClintock.[237] She also observed that spontaneous breakage is unusually high in telocentric chromosomes. High spontaneous fragmentation has also been reported in *Melandrium* plants showing a high degree of asynapsis (Warmke[357]).

2. *Submicroscopic, Cytochemical, and Cytophysiological Factors Influencing Sensitivity.* The availability of a number of chemical substances apparently can have a very definite effect on the reaction of chromosomes to irradiation. Iron and uranium salts, added to the food of *Drosophila*, caused a large increase in X-ray induced mutations.[28] D'Amato and Gustafsson[76] have studied the combined effect of X rays and various chemicals (colchicine, uranyl nitrate, hydrogen peroxide, and $Fe_2(SO_4)_3$) on the mutation rate in barley. They found that almost all pretreatments enhanced the mutation rate. A significant exception was potassium cyanide (0.01 mol.), which reduced the mutation rate well below that of the less concentrated solutions (.001 and .0001), as well as below that of the dry control series. "This

result is of profound interest, since it indicates that an increase of chromosome aberrations—translocations, inversions, deficiencies—does not necessarily raise the amount of detectable mutants. It argues against the view that visible mutations are nothing but chromosome rearrangements or, at least, that they are always connected with the origin of chromosome disturbances. In this special case, we conclude that concentrated solutions of potassium cyanide increase the rates of chromosome breakage, although the genic material does not react with an increase of visible mutants."[76] These data support Moore's[254] conclusion that "breakage is a phenomenon of the matrix and mutation a function of the genes, which lie in the chromonemata." He would, therefore, not expect mutation rate to equal or parallel breakage rate. Gelin,[121] however, concluded that X-ray induced chromosomal disturbances are paralleled by mutation frequency. The results of D'Amato and Gustafsson[76] with colchicine pretreatment are also of special interest because of an apparent qualitative, as well as quantitative, effect on the mutation process.

An increase in sensitivity of man (skin), mice, *Clonorchis*, and *Chaos chaos* has been noted following treatment with or exposure to bergamot oil,[269] methylcholanthrene,[252] gentian violet,[54] and neutral red,[185] respectively. A decrease in sensitivity of *Paramecium* was observed following the addition of cholesterol to the medium (Levin and Piffault[208]).

Anoxia has been shown (Crabtree and Cramer,[68] Mottram,[256] Collodi,[59] and Schrek[301]) to reduce X-ray damage. Changes in sensitivity of cancer cells to radium emanations can be induced by altering the respiratory mechanism of the cells with hydrocyanic acid, cold, and anaerobiosis (Crabtree and Cramer[67]). Synthesis of desoxyribose nucleic acid in liver slices has been shown by Mann and Gruschow[217] to be dependent upon the presence of oxygen. This diminution of X-ray effect under low oxygen tension may be significant in explaining sensitivity changes in *Trillium* chromosomes. Erickson[99] has shown that oxygen consumption of *Lilium* anthers is low during meiotic stages and postmeiotic mitosis, but high between these stages. In *Trillium*, oxygen uptake also falls off sharply in microspore prophase (Stern and Kirk[330]). This is the reciprocal of X-ray sensitivity of these stages in *Trillium* and hence it would appear, during a period of lower consumption of oxygen by the cells, that a higher partial pressure would exist intracellularly, and this would tend to increase sensitivity to X-ray fragmentation, as shown by Thoday and Read.[344]

The state of hydration will also affect mutation rate[184] or chromosome breakage.[135, 121, 263, 271, 272, 336] Dehydration of grasshopper eggs increases sensitivity in early stages but causes a decrease in later stages (Goodrich[123]).

Large changes in permeability have been noted in *Trillium* microspores by Stern.[329] He found that "permeability reaches a maximum value somewhere near the end of prophase or in early metaphase." This coincides with the stage of high X-ray sensitivity (see also p. 1522).

Stubbe[332] has shown that plants deprived of N, P, or S gave a significantly higher mutation rate following radiation than did irradiated control normal plants. In contrast, results with *Drosophila* showed a lower mutation rate

following starvation.[141] Treatment of *Drosophila* with diethyl ether,[142] or with pilocarpin, caffein, sea-water, and progynon[88, 89] increased their sensitivity to X-ray induced mutation. Benzpyrene treatment of *Drosophila* lowers the mutation rate[106] but increases the sensitivity of *Vicia* to X rays.[66] Marquardt and Ernst[221] found that X-ray induced mutation rate is much higher in young than in mature pollen grains of *Antirrhinum majus*. In *Drosophila* sperm, however, lethals are more frequent when old sperm are irradiated than when young sperm are given the same dosage (Offermann,[268] *cf*. also Kaufmann[174]).

A specific Mendelian factor has also been shown to increase the susceptibility of dormant seeds to X-ray injury.[309] This is not too surprising, since both gene mutation and spontaneous chromosome aberration have been shown to be under genic control,[19, 282, 166, 263] and X-ray sensitivities of plant genera differ widely (Johnson[165]). These differences may be partially due to differences in lengths of mitotic cycles. Koller [186] has interpreted his results to mean that the amount of radiation-induced damage depends to a large extent on the length of the life-cycle of the cell relative to the dosage-rate (*cf*. Haldane[138]).

A change in radiosensitivity, following exposure to ammonia[223] and various penetrating acids and bases,[374] has been attributed to a change in pH of the chromosome. It has also been shown that "more of the electrons produced in the surrounding medium reach the virus particles when the latter carry a net positive charge than when the charge is negative."[228] Zirkle[374] has suggested that maximum radiosensitivity may coincide with maximum flocculatility of proteins kept near their isoelectric point during irradiation.

Sax[292] found a lower break frequency in acentric than in centric chromosomes. He suggested that the difference in torsional stress due to lack of centromere might be responsible. An alternative explanation might be based on cytochemical differences between the two types of chromosomes, since certain changes are indicated by staining differences, especially where acentric fragments are left out in the cytoplasm at telophase. It is not known, however, how soon after the initial breakage such changes would become of significance.

An accumulation within the cell of mineral salts or other substances of high atomic number would be expected to increase the absorption of X rays somewhat and, therefore, possibly the amount of ionization in nearby cells or tissue. Cathie[50] has found that the inorganic ash content of a malignant tumor is indicative of its radiosensitivity, and Scott[304] has shown that the concentration of mineral salts in chromatin is most noticeable at late prophase and metaphase (stages of high sensitivity). This agrees with Cathie's[50] observation that in sensitive tumors the ash is scattered throughout the nucleus, while in resistant forms it is in the nuclear membrane. Engstrom,[98] also, has found a correlation between ash content and u.v. absorption in cell nuclei, indicating the association of mineral salts with nucleic acid or nucleotides. Calcium is present within the nucleus and attached to the nucleoprotein in concentrations nine times greater than in

an equivalent volume of cytoplasm.[164] Iron has also been reported in association with nucleic acid[127] and with nucleoprotein.[20] The addition of 0.35 M K citrate has been found to inhibit the retarding action of X rays on cell division in *Arbacia* eggs, but radiosensitivity was not affected by increasing the Ca or Mg content of the sea-water (Wilbur and Recknagel[366]).

A high X-ray resistance of certain plant seeds has been attributed to a high fat content.[136] Small differences in fairly large seeds might be explained on the basis of the low effective atomic number of fat, as shown experimentally by Spiers.[325]

The question of whether the irradiation produces an effect directly on the nucleus or indirectly through the cytoplasm has been investigated by Henshaw[146] and others. Henshaw irradiated normal, nucleated, and non-nucleated *Arbacia* eggs and found a delay in cell division in nucleated cells but no delay in non-nucleated fragments. He concludes that the delay is due to an effect produced directly in the nucleus by irradiation. Similar conclusions were reached by Vintemberger[350] and Zirkle[373] using frog eggs and fern spores, respectively. More recently, Petrova[273] has shown that the nucleus of *Zygonema* is about 700 times more sensitive to radiation than the cytoplasm. In contrast to these results, Duryee[93] has found that isolated nuclei of frog eggs showed no effect with 50,000 r, while 1000 r was sufficient to damage nuclei in intact cells.

There is strong evidence that various kinds of radiation cause a release of calcium in the cytoplasm (references in Heilbrunn and Mazia[144]). It has been suggested that neutralization of the normal negative charge of the chromosome by the calcium ion might cause the observed stickiness and clumping which often follow exposure to radiation (Heilbrunn,[143] p. 658). The same author also points out that "if calcium from the cytoplasm were responsible for chromosomal changes in irradiated cells, then it might well be expected that radiation would be most effective at times when the chromosomes are free in the cytoplasm or when the nuclear membrane is especially permeable, as it may well be just before breakdown" (p. 658).

A thorough review of literature on cytochemistry and submicroscopic structure will not be attempted here. A condensed summary of some important aspects will be given (for more detailed information, see the reviews of Brachet 1944; Frey-Wyssling, 1948; Greenstein, 1944; Gulick, 1941, 1944; Hevesy, 1948; Huskins, 1942; Kossel, 1928; Mirsky, 1943; Schmitt, 1944; Schultz, 1944; Stern, 1947; Waddington, 1940; Wrinch, 1940; and various papers in Biol. Symp. 10, 1943; Symp. Soc. Expt. Biol. I Nucleic Acid, 1947; and Cold Spring Harbor Symp. Quant. Biol., 12, 1947).

The main constituents of chromosomes are desoxyribose nucleic acid (DNA) and one or more basic proteins. A small amount of ribose nucleic acid (RNA) may also be present[31, 245] and, according to some authors, small amounts of lipids (*e.g.*, Hirschler,[152] Serra[306]). Protamine apparently replaces the more complex protein in certain cases. Histone is present in fairly large amounts in most chromosomes. A number of other proteins have been isolated from the nucleus but there is, as yet, little agreement upon their characteristics or relative amounts.

The protein or polypeptides are considered to form a framework (residual chromosome, Mirsky,[245] Mirsky and Ris[246]) upon which, or in which, varying amounts of nucleic acid and possibly other proteins are attached or imbedded. There is still disagreement concerning the relative importance of the protein and nucleic acid components in gene function. Certain enzymes may be present within the chromosomes, as a number have been isolated from nuclei[90, 32] or demonstrated, cytochemically, to be located either on the surface or inside the chromosome.[191, 77, 245, 275] Unfortunately, the nature of the complex between nucleic acid and the protein is not fully understood,[27] nor is there agreement concerning the arrangement of the nucleic acid, nucleoprotein, and protein molecules in the chromosome (see review by Frey-Wyssling[116]). Our knowledge of ultra-structure, or changes in ultra-structure at different stages, is likewise inadequate, and attempts to explain changes in radiosensitivity in terms of ultra-structure would seem premature. We are, therefore, limited to a consideration of gross changes in relative amounts of measurable components (protein, nucleic acid, ash content, *etc.*) present in the chromosome or nucleus at various times or various stages. Even here, the data available are rather inadequate and there is considerable disagreement both as to chemical structure at various stages and concerning the nature and degree of the changes which occur.

DNA has been considered to be present in highest concentration at or near metaphase by a number of authors.[44, 79, 284a] A protective effect of the nucleic acid in preventing breakage has been suggested.[297, 336, 81] Since metaphase is a stage of high sensitivity, however, the nucleic acid can only "protect" in the sense that immediate breakage is low or nil. The ultimate breakage, however, is high. The high sensitivity of the metaphase stage to X-ray breakage suggested to the author[317] that the high concentration of DNA might be related to high sensitivity and, conversely, that a low concentration might be related to a low sensitivity. The recent results of Ris[283] and Sparrow and Rosenfeld (unpublished) indicate, however, that that there is little change in the amount of DNA after late prophase of meiosis and mitosis. If these results are confirmed, it would seem that DNA concentration could only be related to sensitivity by means of an internal shift within individual chromosomes, rather than to a large change in total amount Such a shift might be represented by a gross visible change, such as the appearance of heterochromatic regions at certain stages, or by a change in submicroscopic structure, such as relative amount of protein or nucleic acid in the interchromeric regions (*cf.* Schultz[303]). Evidence in support of the latter is the rather low sensitivity of early meiotic prophase chromosomes of *Trillium*, in which the interchromomeric regions apparently contain little or no DNA.

Highly active protein and nucleic acid synthesizing systems have been demonstrated in young or malignant tissues and in actively working glandular or nerve cells (for references see Hevesy, 1948). On the basis of evidence indicating that nucleic acid metabolism is disturbed following irradiation, Hevesy[149] has suggested that cells or tissues are highly radiosensitive if nucleic acid synthesis is very active and, conversely, that resting cells are

radio-resistant because of their low requirements in this respect. It had previously been suggested that the high radiosensitivity of tumor cells might be associated with their high content of DNA.[317]

Depolymerization of RNA, followed by enzymatic disintegration of nucleoproteins to simple compounds, occurs during telophase, according to Serra.[305] Possibly this change in physical state may be associated with a change in sensitivity. The physical configurations of molecules in artificial pepsin-albumin films have been found to influence X-ray sensitivity (Mazia and Blumenthal[234]).

A relationship between the condition of mitochondria and sensitivity of cells to X and γ rays has been suggested by Joyet-Lavergne.[167] It seems possible that here, too, the nucleic acids may be involved, since mitochondria contain large amounts of nucleic acid (Schneider[299]).

Changes in type and kinds of protein in the chromosomes during the nuclear cycle also occur, according to some investigators. Metaphase chromosomes are considered by Caspersson[43, 44] to be composed of histone, whereas the euchromatic portions of the chromosomes in the interphase nucleus consist largely of the complex type protein, while the heterochromatic regions retain their histone. Since positively heterochromatic regions and metaphase type chromosomes are somewhat similar in appearance, and both highly radiosensitive, one might suspect that the presence of histone (or the absence of complex type protein) is somehow responsible for their greater breakability. Conversely, one might expect that the presence of a complex type of protein would render a chromosome less readily broken and possibly facilitate rejoining of those broken ends which actually do appear.

3. *Effects of Mitotic Inhibitors, Mitotic Poisons, and Miscellaneous Substances on Sensitivity.* Since the sensitivity of various stages of nuclear division differs greatly, it is obvious that any substance or agent altering the normal percentage of the various stages (*cf.* Chalkley[52]) should have an effect upon the overall sensitivity of a tissue or an organ or upon a group of single-celled organisms. Enzyme inhibitors (*e.g.*, carbamates) or substances known to attack nucleoproteins or to effect nucleoprotein metabolism[94] would also be expected to have an effect on sensitivity.

Acridines, mustard gas, nitrogen mustards, methylcholanthrene, hydroquinone, and quinone diimine are all known to attack the nucleoprotein system.[126, 125, 231, 130a, 97, 233] Of these, nitrogen mustard appears to have an effect on radiation-induced mutations, as recently reported by Swanson and Goodgal.[339]

Other compounds, which, by themselves, are known to induce chromosome aberrations, would be expected to enhance radiosensitivity. These include various carbamates, allyl isothiocyanate,[12] paraquinone, pyrogallol, hydroquinone, pyrocatechol,[207] 2-methyl-1,4-naphthoquinone,[240] and saponin.[36a]

Colchicine, acenaphthene (references in Levine[209]), and a vitamin K substitute[251] have been shown to increase sensitivity, presumably by increasing the number of "metaphases." A decrease in the number of chromatid

aberrations, following combined colchicine and X rays, was attributed by Brumfield[35] to a reduction in chromosome movement. An effect of colchicine on chromosome mechanics (crossing-over) was previously reported.[315]

Other mitotic poisons or mitostatic agents which cause unusual accumulations of certain mitotic stages include 1,4-benzoquinone, 1,4-naphthoquinone, 9,10-phenanthroquinone, stilbestrol,[205] protoanemonin,[100] and various other compounds having colchicine-like activity (*cf.* Levine,[209] MacCardle[215]).

Various other substances which might be expected to alter sensitivity are coumarin and parasorbic acid,[64] vitamin A,[161] urethane (ethylcarbamate),[267, 137, 216] ethyl phenyl carbamate, isopropylphenylcarbamate and B-chloroethylcarbamate,[94] aromatic diamidines, podophyllin and its various fractions,[65] inositol, d-desthiobiotin, avidin concentrate,[179] various naphthalene derivatives, trypaflavine and a number of other acridine compounds, dimercaptopropanol (BAL), sodium cyanate, potassium thiocyanate,[94] pyridine, thiouracil, thiourea,[75] certain nitro- and halo-phenols,[57] various antibiotics, such as penicillin, streptomycin, atabrin, and sulfonilamide,[110] certain porphyrins and metalloporphyrins,[109] and a number of pteridines.[264]

The effect of specific hormones in stimulating cell division in particular organs or glands has also been considered as a possible method of increasing sensitivity.[53] Other growth stimulating substances should also have an effect. Changes in chromosome size in mice treated with testosterone propionate[23] might also alter their radiosensitivity. Plant hormones which increase the number of cell divisions or induce otherwise resting cells to divide[160] would also be expected to increase susceptibility to radiation damage. Increased mitotic proliferation in hemopoietic tissue induced by phlebotomy or by acetyl phenylhydrazine, however, apparently caused a decrease in radiosensitivity.[163] The reason for the decrease is not clearly understood. The increased number of divisions, as well as the longer duration of metaphase in tumor tissue,[210] would also be factors effecting an increase in sensitivity.

4. *Effect of Physical Factors on Radiosensitivity.* A number of physical factors over which the organism may have no control are known to have an effect on radiosensitivity. Forssberg[112] has found that a magnetic field of 6,000 Gauss increased the lethal effects of X rays on *Drosophila* eggs. This is not unexpected, since a magnetic field alone has been reported to have an adverse effect on tumor growth (Lenzi[206]). He has also found that a magnetic field seems to cause an accumulation of anaphase stages. This is a stage of medium high sensitivity to X rays.

Temperature, or temperature shock, has also been shown to influence radiation effect, as measured by a number of methods, including protein denaturation,[56] mutation,[181,312] chromosome breakage,[243, 295, 202, 171, 294, 105, 312] and subsequent growth or development.[86, 63, 128, 9, 178] Sax[289] has suggested that, at high temperatures, fusion of broken chromosomes is accelerated, while at lower temperature fusion is delayed. Both low and high temperatures apparently result in morphological and chemical changes in the chromosomes which might alter their reaction to irradiation (Wilson and Boothroyd,[367] Resende[279, 280]).

Sax[293] has investigated the effects of centrifugation during irradiation and found an increase in chromosome aberrations in *Tradescantia*. An increase in radioresistance in the skin of patients has been obtained with compression,[104] and also in artificial albumin-pepsin films (Mazia and Blumenthal[234]).

The combination of various radiations, either simultaneously or one after another, has sometimes given unexpected results. Ultraviolet and infrared have been found to alter the effects of X rays on chromosome breakage or reunion in *Drosophila*[172, 176, 177] and *Tradescantia*[337, 340] and on mutation rate in spores of *Aspergillus* (Swanson[338, 341]). Kaufmann and Gay[175] suggest "that near infrared radiation, when used as a supplementary treatment, is effective in increasing the frequency of chromosomal rearrangements by facilitating recombination, presumably at the expense of restitution, among the ends of chromosomes broken by ionizing radiation." The combined effects of two ionizing radiations (γ rays and fast neutrons) have been found to be non-additive by Mitchell.[250] An increase in tissue sensitivity to X rays has been noted following pretreatment with ultraviolet, high-frequency alternating current, and, possibly, by direct electric current (Carty[42]).

Ultrasonic treatment, combined with X rays, has been shown to increase the number of chromosome aberrations (Conger[61]). Ultrasonics alone can induce cytogenetic changes,[356] but, in Conger's experiments, the sonic treatment used did not produce aberrations, presumably because of the lower frequency used.

A considerable amount of work has been done on the relative biological effects of the different radiations or different wave-lengths, but a detailed consideration of this aspect of sensitivity is not considered appropriate here. For information on this topic, the various reviews mentioned in the beginning of this article should be consulted.

VI. Mechanism of Radiation Effect, Chromosome Breakage, and Mitotic Inhibition

In order to understand how ionizing radiations produce their biological effect, it would be necessary to know not only the effect they have on individual morphological components of the nucleus and cytoplasm, but also how the radiations produce their chemical effects, and how the chemical changes are responsible for the biological effects.

The energy of ionizing radiations, applied in the form of photons, photoelectrons, or fast electrons, causes excitation or ionizations in a proportion of the atoms or molecules present in the body irradiated. Such molecules (or atoms) are highly reactive and are called activated molecules. In the presence of these activated molecules, certain chemical reactions take place which would otherwise occur slowly or not at all (references in Arnow,[11] Clark,[54a] Lea[201]). Isomeric changes which have been suggested as a mechanism of mutation[301a] may also be induced. In aqueous solutions, ionizing radiations produce H_2O_2 (references in Lea[201]) and H and OH radicals (Weiss,[358] Lea,[199] Burton,[36] and Dainton[71]). The presence of H_2O_2, OH radicals, activated molecules, and a complex mixture of other damaged or partially degraded molecules is presumably the initiating factor in altering

the normal biochemical or physiological processes of the cell. The manner in which excitations or ionizations can cause molecular disturbances or rearrangements which ultimately lead to physical breaks or gene mutations is not understood (see Allsopp,[4] and Allsopp and Catcheside[5]). We know very little about the complex chain of events which connects the initial activation with the definitive result.

Hydrogen peroxide is known to be produced by ionizing radiation under certain conditions. In some cases, a portion of the radiation effect can be inhibited by the presence of catalase (Barron *et al.*[14]). There is also evidence that H_2O_2, added to the medium, can have effects similar to certain effects of radiation.[284, 102, 372]

Since we are concerned here mainly with the mechanism of gene mutation and chromosome breakage, it is logical to look for effects of radiation on the main chemical constituents of chromosomes, *i.e.*, proteins and nucleic acid, or their building blocks (polypeptides, amino acid residues, purines, pyrimidines, nucleotides, polynucleotides, pentose sugar, *etc.*), or on associated enzyme systems (see p. 1532).

Denaturation and flocculations of proteins and depolymerization of nucleic acid (or nucleoprotein) fibers represent the gross physical effect (*in vitro*) of both ultraviolet and ionizing radiation. The exact nature of the changes in molecular structure which occur in these cases is not too well understood (see Arnow,[11] Neurath *et al.*,[269] McLaren,[238] and Taylor, *et al.*[342]). In some cases, physical degradation is known to occur (see TABLE 5). In other cases, biological activity (or biochemical reactivity) is impaired or inhibited (see TABLE 6 and, for more detailed information on photo- or radiochemical effects on various molecules or systems, see Arnow,[11] Clark,[55] Carpenter,[41] Weiss,[358] Frilley,[119] McLaren,[238] Geise,[122] and Stein and Weiss[327]).

Since various proteins can be denaturated and precipitated by ultraviolet and ionizing radiations, it would seem logical that the inactivation of hormones and enzymes, or structural changes in genes (mutation), may be related to protein denaturation. It has been shown that the specific lytic power of bacteria can be destroyed by the alteration of a single molecule of nucleoprotein nature,[196] and that the combination of both nucleic acid and protein is necessary for virus multiplication (Markham *et al.*[219]). Since radiations are known to cause degenerative changes in both proteins and nucleic acids, alone or in combination, it would seem reasonable to assume that such changes may somehow be responsible for both chromosome breakage and mutation.

Mutations produced by ultraviolet have been considered by several investigators to be caused by an absorption process in nucleic acid (*e.g.*, Stadler and Uber[326]). Recently, however, a case has been found which suggests that protein rather than nucleic acid absorption may be involved (McAulay and Ford[235]). The radiation-induced changes in nucleoprotein or its components may be in some cases a direct effect of the ionizations or excitations on the molecule in question. It seems more likely, however, that, in the majority of cases, the effect is indirect, beginning with ionization or excitations in molecules or atoms in the immediate vicinity of the gene

or chromosome. Some spreading of the effect would occur (*cf.* Muller,[257] Lea,[201] Kotval and Gray[190]). There is now good evidence that various chemical substances can have effects that very closely simulate those of ionizing radiations (*e.g.*, Auerbach, Robson, and Carr,[134] Rapoport,[277] Darlington and Koller[80]). This provides support for the idea that a chemical substance (or substances), produced by the irradiation, could cause the chromosomal disturbance, rather than the ionizations causing the effect directly (Allsopp and Catcheside[5]).

TABLE 5

EFFECT OF VARIOUS RADIATIONS ON PHYSICAL PROPERTIES OF SUBSTANCES IRRADIATED*

Substance irradiated	*Change(s) induced*	*Radiation used*	*References*
Protoplasm (*Arbacia* egg)	viscosity increased	X rays	Wilson, 1936
Jelly (*Arbacia* egg)	pH slightly decreased and other changes	X rays	Evans *et al.*, 1941
Cytoplasm	viscosity (increase or decrease)	various	Heilbrunn and Mazia, 1936
Gelatin	viscosity reduced	X rays	Woodward, 1932, and Sokolov, 1940
Agar (dry)	viscosity reduced (in sol.)	X rays	Kerston and Dwight, 1937
Apple pectin (dry)	viscosity reduced (in sol.)	X rays	Dwight and Kerston, 1938
Synovial fluid	viscosity reduced	X rays	Ragan *et al.*, 1947
Na thymo-nucleate	viscosity reduced	X rays	Sparrow and Rosenfeld, 1946; Taylor *et al.*, 1948
Nucleohistone	birefringence reduced	X rays	Sparrow and Rosenfeld, 1946; Taylor *et al.*, 1948
Na thymonucleate	viscosity reduced	u.v.	Greenstein and Jenrette, 1941
Various proteins	denaturation, unfolding, splitting (viscosity increase), change in absorption curve, solubility, pH, surface tension, odor, color, *etc.*	u.v. and ionizing radiations	Sanigar *et al.*, 1942 Davis *et al.*, 1942. Other references in Arnow, 1936; Clark, 1936; Fricke, 1938; McLaren, 1947
Acrylonitrite	polymerization	X and γ rays	Dainton, 1947

* See Table 6 also.

If we assume that H_2O_2 is somehow involved in causing chromosomal disturbances, the reported viscosity change (depolymerization?) of thymonucleohistone by H_2O_2 (and vitamin C) may be pertinent (Robertson *et al.*[284]). This is in agreement with Lea,[198] who contends that chromosome damage is brought about through the chemical effect of the ionizations. He has also shown that inactivation of viruses in dilute aqueous solution is mainly indirect, *i.e.*, due to ionization in the water, while inactivation of dry viruses is due to ionization within the virus particle.[198] In the latter case, higher doses are required for inactivation.

DNA and DNP both form highly viscous solutions, indicating a high degree of molecular asymmetry. Exposure to ultraviolet,[132] X rays,[321] or

TABLE 6

EFFECTS OF RADIATIONS ON SELECTED SUBSTANCES OF PHYSIOLOGICAL SIGNIFICANCE*

Substance irradiated	*Change(s) induced*	*Radiation used*	*References*
Gonadotrophin	inactivated	X rays	Hochman *et al.*, 1947
Acetylcholine	inactivated	X rays	Dale, 1943
Plant auxin, phyllocaline	inactivated	X rays	Skoog, 1935; Smith and Kersten, 1942
Glutathione	destroyed	u.v.	Coldwater, 1930
		X rays, γ rays	Hammett, 1932
			Woodward, 1933
			Kinsey, 1935
Vitamin C	inactivated	X rays	Brues *et al.*, 1940
			Anderson and Harrison, 1943
Thiamine chloride	partially destroyed	β and γ rays	Dunlap and Robbins, 1943
Pepsin	inactivated	u.v., β, γ rays	Northrop, 1933–34
Phosphatase	inactivated	u.v. and X rays	Albers, 1940
Ribonuclease	inactivated	X rays	Lea (1947c, p. 38)
Catalase	inactivated	X rays	Forssberg, 1946
Other enzymes	inactivated	u.v. and ionizing radiation	many references, see text
Haemocyanin	molecules split into 2 halves	α rays	Svedberg and Brohult, 1939
Haemoglobin	made heterogenous	α rays	Svedberg and Brohult, 1939
Toxins, venoms	inactivated	u.v. and ionizing radiation	Carpenter *et al.*, 1942
			Efrati *et al.*, 1947
			other references in in Brooks, 1936
Antigen film	inactivated	α rays	Rothen, 1948
Fertilizin	inactivated	u.v. and X rays	Metz, 1942
Paramecium (mating types)	no mating reaction with dosage 500,000 r to 1,000,000 r, symbiotic zoöchlorellae destroyed	X rays	Wichtermann, 1948
Amino acids	liberation of NH_3 change in u.v. absorption	u.v. and X rays	Allen *et al.*, 1937
Caprilic, lauric, and palmitic acids	CO_2, CO, H_2, H_2O and lower fatty acids produced	deuterons α rays	Honig, 1946
Hyaluronic acid	viscosity reduced	X rays	Ragan *et al.*, 1947
Sulphathiazole and Sulphonamide	destroyed	X rays	Tchaperoff, 1943
3,4-Benzypyrene	produces carcinogenic compounds	u.v. 2537Å	Allsopp, 1946
Benzene	OH radicals	X rays	Stein and Weiss, 1948
Benzoic acid	oxidation		
Benzene	OH radicals, catechol formed, benzene ring opens	neutrons and γ rays	Stein and Weiss, 1948
Benzoic acid			

* See also Table 5.

nitrogen mustards[126] induces a progressive fall in viscosity which has been attributed to depolymerization of the long fibrous macro-molecule. Liquefaction of gelled nucleoprotein by X rays has also been reported by Errera.[101] Such gel-sol transformation within the chromosome might be related to the initiation of breakage (*cf.* Kopac[188]). Errera also found that if intact cells were irradiated, and the nucleoprotein then extracted, the latter formed liquid solutions instead of gels. However, von Euler and Hahn[352] were unable to detect any change in nucleoproteins following irradiation (65,000 r) of isolated calf thymus nuclei. An *in vivo* effect of radiation on the viscosity of the cytoplasm has also been observed (Northen and MacVicar,[265] Wilson,[368] for other references, see Heilbrunn and Mazia[144]).

It has been estimated that an average of seventeen ionizations is required to cause a chromosome break in *Tradescantia* (Lea and Catcheside[202]). Estimates of the probable number of polypeptide chains or of DNA molecules in a cross section of a chromonema have been made. Admittedly, such estimates are very crude, but, nevertheless, it is apparent that the number of molecular fibres in a chromosome must be of the order of several thousand. It would therefore appear that the ionizations cannot rupture a sufficient number of molecules directly to result in a chromosome break unless a chain reaction is involved, or unless a single ionization can affect many molecules (*cf.* Muller,[257] Latarjet[196]). We might suspect, therefore, either that the ionizations act as a trigger, or that local damage, *e.g.*, protein denaturation, DNA depolymerization, or rupture of an interfacial membrane, may provide a locus for intracellular enzymes to attack, or for other degenerative processes to occur, and that these would eventually cause a break. In this connection, the observation that alkaline phosphatase acts on depolymerized nucleic acid but not on polymerized nucleic acid is of considerable interest (Krugelis[192]). These results suggest that if depolymerization is initiated by radiation, further breakdown might then be carried on by the phosphatase.

Further evidence in support of such an hypothesis is the observation of von Euler and Hevesy[353] that the enzymatic activity of nuclease (and catalase) increased appreciably after 3000 r. An alternative mechanism might be the inactivation of one or a few molecules in or around the gene itself. Once the gene is sufficiently damaged, the chromosome would no longer be able to maintain its organization in that particular region and breakage would occur. Markham, *et al.*,[219] have recently shown that the presence of combined nucleic acid (*i.e.*, nucleoprotein) is necessary for virus multiplication and that, in every case where nucleic acid is liberated from the protein, the latter is denatured. It would, therefore, appear that if such changes can be induced by radiation they would completely alter the physiological activity of any molecules associated with gene metabolism or function, and this might indirectly interfere with the reproduction or the physical continuity of the gene string. Similarly, the effect of the recoil associated with radioactivity of an atom (*e.g.*, P^{32}, S^{35}, or C^{14}) incorporated into the molecular structure of the chromosome could have a very drastic

effect on the structure or physiological function of the molecule involved. The associated transmutation, *e.g.*, of P^{32} to sulphur, might also be expected to cause a molecular change with subsequent biological effect. The effect of recoil or transmutation can probably result in chromosome breakage or mutation(Giles,[124] Powers,[274] Arnason *et al.*,[10] and Rubin[286]).

The phenomenon of mitotic inhibition immediately following radiation is probably only indirectly related to mutation and chromosome breakage. Inhibition of the first mitosis following irradiation is probably due to a physiological disturbance resulting directly from the irradiation (enzyme inactivation, *etc.*), while the long-term effect is more likely caused by mutation or loss of genetic substance by chromosome fragmentation.

Destruction of auxin by X rays has been reported by Skoog[307] and by Smith and Kersten.[308] Skoog attributed the temporary inhibition of plant growth following radiation to such destruction. Growth returns to normal after the plant has had sufficient time to produce a new supply of the hormone.

More recently, von Euler[351] has found that the growth inhibiting action of X rays (alone or in combination with colchicine) is increased if the germinating barley seeds are washed. The inference is that the washing removes a portion of the natural growth-promoting substance, the loss of which increases radiosensitivity.

Various investigators have found high-frequency or ultraviolet radiation to have an effect on nucleic acid metabolism (Mitchell,[247, 248, 249] von Euler and Hevesy,[354] Hevesy,[149] other references in Hevesy[150]), which is considered by Caspersson,[45] Greenstein,[131] and others to be related to protein synthesis. If the nucleic acid content of chromosomes is increasing during prophase, and must reach a certain critical level before division can occur, any interfering mechanism would be expected to hold up division until synthesis could recover from the temporary damage or delay. Lindegren[212] has suggested that a coating of metaphosphate on the surface of yeast chromosomes is required before they can divide, and Wiame[364] considers that the metaphosphate may be a source of energy through its energy-rich phosphate bonds. Spiegelman and Kamen[324] suggest that the nucleic acid serves as the agent which funnels energy into the protein-synthesizing mechanism. Any interference with the formation of the necessary coating would be expected to cause mitotic inhibition.

Specific metabolic processes could be blocked or partially inhibited by inactivation of the enzyme or enzymes concerned. A number of studies have shown that the activity of a large number of enzymes can be reduced by exposure to ionizing or ultraviolet radiation (Northrop,[266] Schomer,[300] Iwatsuru and Nanjo,[162] Albers,[1] Miyosi,[253] Tytell and Kersten,[347] Dale,[73, 74, 75] Martin,[229] Forssberg,[113, 114] Anderson,[7] and Mazia and Blumenthal[234]). Doses as low as 5 r have been shown to have an effect on catalase (Forssberg[115]). Doses in the therapeutic range (25–500 r) have also been shown to have relatively large effects on certain sulfhydryl enzymes through the oxidation of the—SH groups by OH radicals and by H_2O_2 (Barron *et al.*[14]). In some cases, inhibition could be reduced by the addition of glutathione or catalase.

Enzymes and viruses in dilute aqueous solution can be "protected" from inactivation by radiation by the addition of various substances, such as protein, gelatin, glucose, nucleic acid, thiourea, *etc.* (Dale,[73, 75] Friedewald and Anderson[118]). Different substances show varying degrees of "protection." If we assume the major effect of ionizing radiation on enzymes to be oxidation by the OH radicals produced, then substances most likely to react with hydroxyl radicals would be expected to have the highest "protecting power."[4] Since nucleic acid "protects" enzymes,[73] it may also "protect" the protein moiety of chromosomes against radiation-induced changes. However, if the OH radicals have an affinity for nucleic acid (as indicated by protective effect), and the OH can depolymerize the DNA, then the depolymerization might initiate the mechanism leading to breakage. (It is known that pH changes cause depolymerization of sodium thymonucleate *in vitro*.[348]) RNA would also "protect," but that present outside the chromosome would not affect breakage unless the OH radicals produced diffuse an appreciable distance. Lea's[199] calculations indicate that they do not.

VII. Summary

Original data on sensitivity changes to X rays during the nuclear cycle in *Trillium erectum* are presented. They show late prophase and metaphase to be highly sensitive to X-ray breakage and early interphase to be of low sensitivity. The tendency of broken ends to rejoin seems to be inversely related to the amount of fragmentation per unit dosage. The general significance of these conclusions is discussed in the light of other work on sensitivity changes during nuclear division. The distribution of chromosome breaks within chromosomes and the relationship of breakage to mutation are also discussed.

The factors affecting nuclear radiosensitivity are considered under the following topics:

(1) Morphological and structural differentiation within the nucleus.
(2) Sub-microscopic, cytochemical, and cytophysiological factors.
(3) Effects of mitotic inhibitors, mitotic poisons, and other miscellaneous substances.
(4) Effects of physical factors.

The individual factors affecting sensitivity are too numerous to mention in a summary and it is exceedingly difficult to draw general conclusions. It appears, however, that the condensed state of the chromosome (late prophase, metaphase, anaphase) is one of high sensitivity to breakage, and that the diffuse stages, such as telophase, interphase, and early prophase are relatively much less sensitive. Possible reasons for these differences are considered, and possible mechanisms, whereby the processes initiated by ionizations produce effective chromosome breaks, are presented and discussed.

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Bibliography

1. Albers, D. 1940. Fundamenta Radiol. **5:** 157–164.
2. Allen, A. J., R. E. Steiger, M. A. Magill, & R. G. Franklin. 1937. Biochem. J. **31:** 195–204.
3. Allsopp, C. B. 1946. Cancer Research **6:** 24–28.
4. Allsopp, C. B. 1948. Brit. J. Radiology **21:** 72–74.
5. Allsopp, C. B. & D. G. Catcheside. 1948. Nature **161:** 1011–1012.
6. Anderson, E. G. 1931. Genetics **16:** 386–396.
7. Anderson, R. S. 1948. Biol. Bull. **93:** 189.
8. Anderson, R. S. & B. Harrison. 1943. J. Gen. Physiol. **27:** 69–75.
9. Anderson, R. S. & H. Turkowitz. 1941. Am. J. Roentgenol. **46:** 537–542.
10. Arnason, T. J., E. Cumming, & J. W. T. Spinks. 1948. Science **107:** 198–199.
11. Arnow, L. E. 1936. Physiol. Rev. **16:** 671–685.
12. Auerbach, C. & J. M. Robson. 1944. Nature **154:** 81.
13. Auerbach, C., J. M. Robson, & J. G. Carr. 1947. Science **105:** 243–247.
14. Barron, E. S. G., S. Dickman, & T. P. Singer. 1947. Federation Proc. **6:** 236.
15. Bauer, H. 1942. Chromosoma **2:** 407–458.
16. Bauer, H., M. Demerec, & B. P. Kaufmann. 1938. Genetics **23:** 610–630.
17. Bauer, H. & J. LeCalvez. 1944. Chromosoma (Z. Zellforsch u. Mikroscop. Anat. Abt. B) **2:** 593–617.
18. Bauer, H. & W. Lerche. 1943. Chromosoma (Z. Zellforsch. u. Mikroscop. Anat. Abt. B) **2:** 482–492.
19. Beadle, G. W. 1932. Z. Indukt. Abstamm-u. Vererbungsl. **63:** 195–217.
20. Berthier, J. 1947. Compt. rend. **225:** 1034–1036.
21. Biesele, J. J. 1944. Cancer Research **4:** 737–750.
22. Biesele, J. J. 1945. Cancer Research **5:** 179–182.
23. Biesele, J. J. & G. Gasic. 1947. Cancer Research **7:** 65–69.
24. Biesele, J. J. & H. Poyner. 1943. Cancer Research **3:** 779–783.
26. Bishop, C. J. 1950. Genetics **35:** 175–187.
27. Bjornesjo, K. B. & T. Teorell. 1945. Arkiv Kemi, Mineral. Geol. **19A** (34): 1–26.
28. Born, H. J. & K. G. Zimmer. 1940. Z. Ind. Abst.-Vererbungsl. **78:** 246–250.
30. Bozeman, M. L. 1943. Genetics **28:** 71.
31. Brachet, J. 1944. Embryologie Chimique. Masson et Cie, Paris.
32. Brachet, J. 1945. Enzymologie **11:** 336–347.
33. Brooks, S. C. 1936. Biological Effects of Radiation. Ed. by B. M. Duggar. **1:** 341–388 (Review) McGraw-Hill. New York and London.
34. Brues, A. M., B. B. Marble, & E. B. Jackson. 1940. Am. J. Cancer **38:** 159–168.
35. Brumfield, R. T. 1943. Proc. Nat. Acad. Sci. U. S. **29:** 190–193.
36. Burton, M. 1947. J. Phys. Cell. Chem. **51:** 611–625.
36a. Burtros, J. 1948. Cancer Research **8:** 221–230.
37. Camara, A. 1940. Scientia Genetica **1:** 339–353.
38. Carlson, J. G. 1941. Cold Spring Harbor Symp. Quant. Biol. **9:** 104–112.
39. Carlson, J. G. 1942. J. Morphol. **71:** 449–462.
40. Carpenter, C. M., H. E. Stokinger, H. Ackerman, & S. L. Warren. 1942. J Bact. **43:** 75–76.
41. Carpenter, D. C. 1940. J. Am. Chem. Soc. **62:** 289–291.
42. Carty, J. R. 1930. Radiology **15:** 353–358.
43. Caspersson, T. 1940. Chromosoma **1:** 562–604.
44. Caspersson, T. 1941. Naturwissenschaften **29:** 33–43.
45. Caspersson, T. 1947. Symp. Soc. Exp. Biol. **1:** 127–151.
46. Catcheside, D. G. 1945. Biol. Revs. Cambridge Phil. Soc. **20:** 14–28.
47. Catcheside, D. G. 1948. Adv. in Genetics **2:** 271-358.
48. Catcheside, D. G. & D. E. Lea. 1943. J. Genetics **45:** 186–196.

49. Catcheside, D. G. & D. E. Lea. 1945. J. Genetics **47:** 25–40.
50. Cathie, I. A. B. 1939. J. Path. Bact. **48:** 1–10.
51. Chalkley, H. W. 1936. J. Morph. **60:** 13–29.
52. Chalkley, H. W. 1951. Ann. N. Y. Acad. Sci. **51**(8): 1303.
53. Chamorro, A. 1948. Compt. rend. soc. biol. **142:** 3–4.
54. Chu, H. J. & C. L. Hsu. 1940. Chinese Med. J. Suppl. **3:** 260–266.
54a. Clark, G. L. 1944. Med. Physics 1345–1348.
55. Clark, J. H. 1936. Biological Effects of Radiation. Ed. by B. M. Duggar. **1:** 303–322. McGraw-Hill. New York and London.
56. Clark, J. H. 1938. Am. J. Roentgenol. **40:** 501–508.
57. Clowes, G. H. A. 1951. Ann. N. Y. Acad. Sci. **51**(8): 1409.
58. Coldwater, K. B. 1930. Proc. Soc. Exp. Biol. Med. **27:** 1031–1033.
59. Collodi, G. 1940. Boll. soc. ital. biol. sper. **15:** 997–999.
60. Conger, A. 1947. Am. J. Botany **34:** 582.
61. Conger, A. 1948. Proc. Nat. Acad. Sci. **34:** 470–474.
62. Conklin, E. G. 1951. Ann. N. Y. Acad. Sci. **51**(8): 1281.
63. Cook, E. V. 1939. Radiology **32:** 289–293.
64. Cornman, I. 1947. J. Exp. Biol. **23:** 292–297.
65. Cornman, I. & M. E. Cornman. 1951. Ann. N. Y. Acad. Sci. **51**(8): 1443.
66. Cottet, P. & W. Minder. 1947. Schweiz, med. Wochsch. **77:** 195–197.
67. Crabtree, H. G. & W. Cramer. 1933. Proc. Roy. Soc., B. **113:** 238–250.
68. Crabtree, H. G. & W. Cramer. 1934. Imp. Cancer Research Fund Sci. Report No. **11:** 75–88.
69. Creighton, M. 1941. J. Exptl. Zool. **87:** 347–369.
70. Crowther, J. A., H. Liebmann, & R. Jones. 1940. Phil. Mag. **29:** 391–399.
71. Dainton, F. S. 1947. Nature **160:** 268–269.
72. Dainton, F. S. 1948. J. Phys. Coll. Chem. **52:** 490–517.
73. Dale, W. M. 1942. Biochem. J. **36:** 80–85.
74. Dale, W. M. 1943. J. Physiol. **102:** 50–54.
75. Dale, W. M. 1947. Brit. J. Radiol. Supp. **1:** 46–50.
76. D'Amato, F. & A. Gustafsson. 1948. Heriditas **34:** 181–192.
77. Danielli, J. F. & D. G. Catcheside. 1945. Nature **156:** 294.
78. Darlington, C. D. 1937. Recent Advances in Cytology. 2nd ed. P. Blakiston. Philadelphia, Pa.
79. Darlington, C. D. 1942. Nature **149:** 66-69.
80. Darlington, C. D. & P. C. Koller. 1947. Heredity **1:** 187–221.
81. Darlington, C. D. & L. F. LaCour. 1945. J. Genetics **46:** 180–267.
82. Darlington, C. D. & P. T. Thomas. 1941. Proc. Roy. Soc. B **130:** 127–150.
83. Davis, B. D., A. Hollaender, & J. P. Greenstein. 1942. J. Biol. Chem. **146:** 663–671.
84. Demerec, M. & U. Fano. 1941. Proc. Nat. Acad. Sci. U. S. **27:** 24–31.
85. Dobson, R. L. & J. H. Lawrence. 1948. Ann. Rev. Physiol. **10:** 479–500.
86. Dognon, A. 1926. Compt. rend. soc. biol. **94:** 466–468.
87. Dognon, A. & L. Gourgerot. 1945. Bull. soc. chim. biol. **27:** 240–244.
88. Dotterweich, H. 1939. Zool. Anz. Suppl. (Verhandl. deutsch. zool. Ges. 41) **12:** 244–253.
89. Dotterweich, H. 1940. Z. Indukt. Abstamm-u. Vererbungsl. **78:** 261–272.
90. Dounce, A. L. 1943. J. Biol. Chem. **147:** 685–698.
91. Draver, B. N. 1947. J. Ind. Hyg. Toxicol. **29:** 196.
92. Dunlap, C. E. & F. C. Robbins. 1943. Am. J. Roentgenol. Radium Therapy **50:** 641–647.
93. Duryee, W. R. 1939. Anat. Rec. **75:** suppl. 144.
94. Dustin, P. 1947. Nature **159:** 794–797.
95. Dwight, C. H. & H. Kersten. 1938. J. Phys. Chem. **42:** 1167–1169.
96. Efrati, E., A. Back, & L. Halbersteadter. 1947. Nature **159:** 715–716.
97. Elmore, D. T., J. M. Gulland, D. O. Jordan, & H. F. W. Taylor. 1948. Biochem. J. **42:** 308–316.
98. Engström, A. 1943. Chromosoma (Z. Zellforsch. u. mikroscop. Anat. Abt. B) **2:** 459–472.
99. Erickson, R. O. 1947. Nature **159:** 275–276.
100. Erickson, R. O. 1948. Science **108:** 533.
101. Errera, M. 1946. Bull. soc. chim. biol. **28:** 472–477.
102. Evans, T. C. 1947. Biol. Bull. **92:** 99–109.
103. Evans, T. C., H. W. Beams, & M. E. Smith. 1941. Biol. Bull. **80:** 363–370.
104. Evans, T. C. & H. D. Kerr. 1943. Am. J. Roentgenol. **50:** 629–635.

105. Faberge, A. C. 1948. Genetics **33:** 104.
106. Fabian, G. & G. Matoltsy. 1946. Nature **158:** 911–912.
107. Fano, U. 1947. Science **106:** 87–88.
108. Fano, U. & M. Demerec. 1944. Med. Phys. **495:** 512.
109. Figge, F. H. J., G. S. Weiland, & L. O. J. Manganiello. 1948. Proc. Soc. Exp. Biol. Med. **68:** 640–461.
110. Flocks, R., O. N. Fellowes, & H. D. Kerr. 1940. Am. J. Roentgenol. **44:** 115–116.
111. Fogg, L. C. & S. Warren. 1940. Science **91:** 528–529.
112. Forssberg, H. 1940. Acta Radiol. **21:** 213–220.
113. Forssberg, A. 1945. Arkiv Kemi, Mineral. Geol. **21A**(7): 1–15.
114. Forssberg, A. 1946. Acta Radiol. **27:** 281–293.
115. Forssberg, A. 1947. Nature **159:** 308–309.
116. Frey-Wyssling, A. 1948. Submicroscopic Morphology of Protoplasm and Its Derivatives. Elsevier. New York.
117. Fricke, H. 1938. Cold Spring Harbor Symp. Quant. Biol. **6:** 164–170.
118. Friedewald, W. F. & R. S. Anderson. 1941. J. Exp. Med. **74:** 463–487.
119. Frilley, M. 1947. Brit. J. Radiol. Suppl. **1:** 50–55.
120. Froier, K., O. Gelin, & A. Gustafsson. 1941. Bot. An. Notiser **199:** 216.
121. Gelin, O. E. V. 1941. Hereditas **27:** 209–219.
122. Giese, A. C. 1947. Quart. Rev. Biol. **22:** 253–282.
123. Giles, N. 1940. Genetics **25:** 69–87.
124. Giles, N. H. 1947. Proc. Nat. Acad. Sci. **33:** 283–287.
125. Gilman, A. & F. S. Philips. 1946. Science **103:** 409–415, 436.
126. Gjessing, E. C. & A. Chanutin. 1946. Cancer Res. **6:** 593–598.
127. Goldstein, B. I. & D. V. Vol'kenzon. 1947. Biokhimiya **12:** 89–96.
128. Goodrich, J. P. 1943. Radiology **40:** 179–187.
129. Goodspeed, T. H. 1929. J. Heredity **2** : 243–259.
130. Goodspeed, T. H. & F. M. Uber. 1939. Botan. Rev. **5:** 1–48.
130a. Gopal-Ayengar, A. R. & E. V. Cowdry. 1947. Cancer Research **7:** 1–8.
131. Greenstein, J. P. 1944. Advances in Protein Chem. **1:** 209–287.
132. Greenstein, J. P. & W. V. Jenrette. 1941. Cold Spring Harbor Symp. Quant. Biol. **9:** 236–254.
133. Gulick, A. 1941. Botan. Rev. **7:** 433–457.
134. Gulick, A. 1944. Advances in Enzymol. **4:** 1–39.
135. Gustafsson, A. 1937. Hereditas **22:** 281–335.
136. Gustafsson, A. 1944. Hereditas **30:** 165–178.
137. Guyer, M. F. & P. E. Claus. 1947. Proc. Soc. Exptl. Biol. Med. **64:** 3–5.
138. Haldane, J. B. S. 1948. Proc. Roy. Soc. (London) B. **135:** 147–170.
139. Haldane, J. B. S. & D. E. Lea. 1947. J. Genetics **48:** 1–10.
140. Hammett, F. S. 1932. Protoplasma **15:** 422–426.
141. Hanson, F. B. & F. Heys. 1933a. Am. Naturalist **67:** 127–134.
142. Hanson, F. B. & F. Heys. 1933b. Am. Naturalist **67:** 419–428.
143. Heilbrunn L. V. 1947. An Outline of General Physiology. 2nd Ed. W. B. Saunders. Philadelphia and London.
144. Heilbrunn, L. V. & D. Mazia. 1936. Biological Effects of Radiation. Ed. by B. M. Duggar. Chapt. XVIII McGraw-Hill. New York and London.
145. Helwig, E. R. 1933. J. Morphol. **55:** 265–311.
146. Henshaw, P. S. 1938. Am. J. Cancer **33:** 258–264.
147. Henshaw, P. S. & D. S. Francis. 1935. J. Cell. Comp. Physiol. **7:** 173–195.
148. Herskowitz, I. H. 1946. Am. Naturalist **80:** 588–592.
149. Hevesy, G. 1945. Rev. Modern Phys. **17:** 102–111.
150. Hevesy, G. 1948. Adv. in Biol. and Med. Physics **1:** 409–454.
151. Hinton, T. 1941. Genetics **26:** 119–127.
152. Hirschler, J. 1942. Naturwiss **30:** 642–644.
153. Hochman, A., R. Black, G. Goldhaber, & F. Sulman. 1947. J. Endocrinol. **5:** 99–102.
154. Hollaender, A. 1946. Ann. Rev. Physiol. **8:** 1–16.
155. Hollaender, A. & C. W. Emmons. 1941. Cold Spring Harbor Symp. Quant. Biol. **9:** 179–186.
157. Holthusen, H. 1921. Pflugers Arch. Ges. Physiol. **187:** 1–24.
158. Honig, R. E. 1946. Science **104:** 27–29.
159. Huskins, C. L. 1942. The Structure of Protoplasm: 109–126. Iowa State College Press. Ames, Ia.

160. Huskins, C. L. & L. N. Steinitz. 1948. J. Hered. **39:** 67–77.
161. Huzita, Y. 1939. Jap. J. Obstetr. Gynecol. **22:** 38–48.
162. Iwatsuru, R. & K. Nanjo. 1939. Biochem. Z. **300:** 429–436.
163. Jacobson, L. O., E. K. Marks, E. O. Gaston, E. L. Simmons, & M. H. Block. 1948. Science **107:** 248–250.
164. Jeener, R. 1946. Compt. rend. soc. biol. **140:** 1337–1338.
165. Johnson, E. L. 1936. Plant Physiol. **11:** 319–342.
166. Jones, D. F. 1940. Am. J. Botany **27:** 149–155.
167. Joyet-Lavergne, P. 1938. Arch. Phys. biol. **15:** 94–95.
168. Kaufmann, B. P. 1939. Proc. Nat. Acad. Sci. U. S. **25:** 571–577.
169. Kaufmann, B. P. 1941. Cold Spring Harbor, Symp. Quant. Biology **9:** 82–92.
170. Kaufmann, B. P. 1943a. Proc. Nat. Acad. Science **29:** 8–12.
171. Kaufmann, B. P. 1943b. Rec. Genet. Soc. Am. **12:** 50.
172. Kaufmann, B. P. 1946a. Genetics **31:** 449–453.
173. Kaufmann, B. P. 1946b. J. Exp. Zool. **102:** 293–320.
174. Kaufmann, B. P. 1947. Am. Naturalist **81:** 77–80.
175. Kaufmann, B. P. & H. Gay. 1947. Proc. Nat. Acad. Sci. **33:** 366–372.
176. Kaufmann, B. P. & A. Hollaender. 1946. Genetics **31:** 368–376.
177. Kaufmann, B. P., A. Hollaender, & H. Gay. 1946. Genetics **31:** 349–367.
178. Kempton, J. H. & L. R. Maxwell. 1941. J. Agr. Research **62:** 203–618.
179. Keresztesy, J. C., D. Laszlo, & C. Leuchtenberger. 1946. Cancer Research **6:** 128–130.
180. Kersten, H. & C. H. Dwight. 1937. J. Phys. Chem. **41:** 687–689.
181. King, E. D. 1947. Genetics **32:** 161–164.
182. Kinsey, V. E. 1935. J. Biol. Chem. **110:** 551–558.
183. Knapp, E. 1935. Z. Indukt. Abstamm-u. Vererbungsl. **70:** 309–349.
184. Knapp, E. & R. Kaplan. 1942. Z. Indukt. Abstamm-u. Vererbungsl. **80:** 501–550.
185. Koehring, V. 1940. Radiology **35:** 229–235.
186. Koller, P. C. 1946. Brit. J. Radiology **19:** 393–404.
187. Koller, P. C. & D. W. Smithers. 1946. Brit. J. Radiol. **19:** 89–100.
188. Kopac, M. J. 1951. Ann. N. Y. Acad. Sci. **51**(8): 1541.
189. Kossel, A. 1928. The Protamines and Histones. Longmans Green. New York.
190. Kotval, J. P. & L. H. Gray. 1947. J. Genetics **48:** 135–154.
191. Krugelis, E. J. 1942. J. Cell. Comp. Physiol. **19:** 1–3.
192. Krugelis, E. J. 1946. Biol. Bull. **90:** 220–233.
193. Kvajevoy, S. J. & R. A. Rassuly. 1935. Compt. rend. acad. sci. U.R.S.S. **4:** 225–229.
194. Lamy, R. J. 1946. Genetics. **48:** 223–236.
195. Langendorf. H. & M. Langendorf. 1931. Strahlentherapie **40:** 97–110.
196. Latarjet, R. 1946. Rev. can. biol. **5:** 9–47.
197. Lavedan, J. 1945. L'Action des Radiations sur la Cellule Normale. Gauthier-Villars, Paris.
198. Lea, D. E. 1946. Brit. J. Radiol. **19:** 205–212.
199. Lea, D. E. 1947a. Brit. J. Radiol. Supp. **1:** 59–64.
200. Lea, D. E. 1947b. Brit. J. Radiol. Supp. **1:** 120–137.
201. Lea, D. E. 1947c. Actions on Radiation on Living Cells. Univ. Press, Cambridge. MacMillan Co., New York.
202. Lea, D. E. & D. G. Catcheside. 1942. J. Genetics **44:** 216–245.
203. Lea, D. E. & D. G. Catcheside. 1945. J. Genetics **47:** 10–24.
204. Lederberg, J. 1946. Science **104:** 428.
205. Lehmann, F. E. 1945. Rev. Suisse Zool. **52:** 342–348.
206. Lenzi, M. 1942. Radiol. Med. **29:** 298–305.
207. Levan, A. & J. H. Tzio. 1948. Hereditas **34:** 250–252.
208. Levin, B. S. & C. Piffault. 1934. Compt. rend. Soc. de Biologie **116:** 627–629.
209. Levine, M. 1951. Ann. N. Y. Acad. Sci. **51**(8): 1365.
210. Lewis, W. H. 1951. Ann. N. Y. Acad. Sci. **51**(8): 1287.
211. Lewitsky, G. & M. Sizova. 1935. Compt. rend. acad. sci. U.R.S.S. **4:** 70-75.
212. Lindegren, C. C. 1947. Nature **159:** 63–64.
213. Loeb, L. 1944. Colloid Chemistry. Reinhold. New York. **5:** 995–1050.
214. Luther, W. 1938. Strahlentherapie **62:** 436–449.
215. MacCardle, R. C. 1951. Ann. N. Y. Acad. Sci. **51**(8): 1489.
216. Macht, D. I. 1948. Proc. Soc. Exptl. Biol. Med. **67:** 237–240.
217. Mann, W. & J. Gruschow. 1949. Proc. Soc. Exptl. Biol. Med. **71:** 658–660.

218. Manton, I. 1945. Ann. Botany **9:** 155–178.
219. Markham, R., R. F. Mathews, & K. M. Smith. 1948. Nature **162:** 88–90.
220. Marquardt, H. 1942. Ber. deut. botan. Ges. **60:** 98–124.
221. Marquardt, H. & H. Ernst. 1940. Z. Botan **35:** 191–223.
222. Marshak, A. 1935. J. Gen. Physiol. **19:** 179–198.
223. Marshak, A. 1938a. Proc. Soc. Exptl. Biol. Med. **39:** 194–198.
224. Marshak, A. 1938b. Proc. Soc. Exptl. Biol. Med. **38:** 705–713.
225. Marshak, A. 1939. Proc. Nat. Acad. Sci. U. S. **25:** 510–516.
226. Marshak, A. 1942. Radiology **39:** 621–626.
227. Marshak, A. & M. Bradley. 1944. Proc. Nat. Acad. Sci. **30:** 231–237.
228. Marshak, A. & W. N. Takahashi. 1942. Proc. Nat. Acad. Sci., **28:** 211–216.
229. Martin, L. 1946. Compt. rend. soc. biol. **140:** 1245–1246.
231. Massart, L., G. Peeters, & A. van Houcke. 1947. Arch. intern. pharmacodynamie **75:** 210–221.
232. Mavor, J. W. 1929. Genetics **14:** 129–159.
233. Mayer, R. L. 1948. Proc. Soc. Exp. Biol. and Med. **68:** 664–668.
234. Mazia, D. & G. Blumenthal. 1948. Proc. Nat. Acad. Sci. **34:** 328–336.
235. McAulay, A. L. & J. M. Ford. 1947. Heredity **1:** 247–257.
236. McClintock, B. 1941a. Genetics **26:** 234–282.
237. McClintock, B. 1941b. Cold Spring Harbor Symp. Quant. Biol. **9:** 72–80.
238. McLaren, D. A. 1947. J. Polymer Sci. **2:** 107–109.
239. Meglitsch P. 1939. J. Parasitol. **25:** 441–442.
240. Meier, R. & B. Shär. 1947. Experentia **3:** 358–366.
241. Metz, C. B. 1942. Biol. Bull. **82:** 446–454.
242. Metz C. W. 1934. Proc. Nat. Acad. Sci. **20:** 159–163.
243. Mickey, G. H. 1938. Genetics **23:** 160.
244. Mirsky, A. E. 1943. Advances in Enzymol. **3:** 1–34.
245. Mirsky, A. E. 1947. Cold Spring Harbor Symp. Quant. Biol. **12:** 143–146.
246. Mirsky, A. E. & H. Ris. 1947. J. Gen. Physiol. **31:** 7–18.
247. Mitchell J. S. 1942a. Brit. J. Exp. Path. **23:** 296–309.
248. Mitchell, J. S. 1942b. Brit. J. Exp. Path. **23:** 309–313.
249. Mitchell, J. S. 1943. Brit. J. Radiology **16:** 339–343.
250. Mitchell, J. S. 1947. Brit. J. Radiology **20:** 368–380.
251. Mitchell, J. S. & I. Simon-Reuss. 1947. Nature **160:** 98–99.
252. Mixer, H. W. & A. Kirschbaum. 1948. Radiology **50:** 476–480.
253. Miyosi, H. 1940. Jap. J. Obst. Gynecol. **23:** 263–283.
254. Moore, W. G. 1934. Genetics **19:** 209–222.
255. Mottram, J. C. 1913. Arch. Middlesex Hosp. **30:** 98–119.
256. Mottram, J. C. 1935. Brit. J. Radiology **8:** 32–39, 643–651.
257. Muller, H. J. 1940. J. Genetics **40:** 1–66.
258. Muller, H. J. 1941. Cold Spring Harbor Symp. Quant. Biol. **9:** 151–167.
259. Muntzing, A. 1941. Kgl. pyscografiske Sallskafels I Lund Forhandlingas, Bd 11, nr. **6:** 1–10.
260. Neurath, H., J. P. Greenstein, F. W. Putnam, & J. O. Erickson. 1944. Chem. Revs. **34:** 157–265.
261. Newcombe, H. B. 1942a. J. Genetics **43:** 145–171.
262. Newcombe, H. B. 1942b. J. Genetics **43:** 237–248.
263. Nichols, C. 1941. Genetics **26:** 89–100.
264. Norris, E. R. & J. Majnarich. 1948. Am. J. of Physiol. **153:** 488–491.
265. Northen, H. T. & R. MacVicar. 1940. Biodynamica **3:** 28–32.
266. Northrop, J. H. 1933–34. J. Gen. Physiol. **17:** 359–363.
267. Oehlkers, F. 1943. Zeit. Indukt. Abst. u. Vererbungls. **81:** 313–341.
268. Offermann, C. A. 1939. Genetics **24:** 81–82.
269. Oppenheim, M. 1947. J. Investigative Dermatol. **8:** 255–262.
270. Packard, C. 1916. J. Exptl. Zool. **21:** 199–212.
271. Packard, C. 1931. Quart. Rev. Biol. **6:** 253–280.
272. Packard, C. 1945. Radiology **45:** 522–533.
273. Petrova, J. 1942. Beih. bot. Centralblatt. Abt. A **61:** 399–430.
274. Powers, E. L. 1948. Genetics (abstract) **33:** 120–121.
275. Rabinovitch, M., L. C. U. Junqueira, & F. T. Mendes. 1948. Science **107:** 322–323.
276. Ragan, C., C. P. Donlan, J. A. Coss, & A. F. Grubin. 1947. Proc. Soc. Exptl. Biol. and Med. **66:** 170–172.
277. Rapoport, I. A. 1947. J. Gen. Biol. (U.S.S.R.) **8:** 359–379.

278. Regaud, C. 1923. Bull. assoc. franc. etude cancer **12:** 482–487.
279. Resende, F. 1944. Portug. Acta Biol. **1:** 9–46.
280. Resende, F., J. Salord, & L. Leite-Rio. 1946. Portug. Acta Biol. **1:** 412–415.
281. Reynolds, J. P. 1941. Proc. Nat. Acad. Sci. U. S. **27:** 204–208.
282. Rhoades, M. M. 1938. Genetics **23:** 377–397.
283. Ris, H. 1947. Cold Spring Harbor Symp. Quant. Biol. **12:** 158–160.
284. Robertson, W. V. B., M. W. Ropes, & W. Bauer. 1942. Proc. Soc. Exptl. Biol. Med. **49:** 697–698.
284a. Roskin, G. 1945. Compt. rend. Acad. Sci. U.R.S.S. **49:** 288–291.
285. Rothen, A. 1948. J. Biol. Chem. **172:** 841–842.
286. Rubin, B. A. 1948. Rec. Genetics Soc. Am. **17:** 55–56.
287. Sanigar, E. B., L. E. Krejci, & E. O. Kraemer. 1939. Biochem. J. **33:** 1–16.
288. Sax, K. 1938. Genetics **23:** 494–516.
289. Sax, K. 1940. Genetics **25:** 41–68.
290. Sax, K. 1941. Cold Spring Harbor Symp. Quant. Biol. **9:** 93–101.
291. Sax, K. 1942a. J. Gen. Physiol. **25:** 533–537.
292. Sax, K. 1942b. Proc. Nat. Acad. Sci. U. S. **28:** 229–233.
293. Sax, K. 1943. Proc. Nat. Acad. Sci. U. S. **29:** 18–21.
294. Sax, K. 1947. Genetics **32:** 75–78.
295. Sax, K. & E. V. Enzmann. 1939. Proc. Nat. Acad. Sci. U. S. **25:** 397–405.
296. Sax, K. & K. Mather. 1939. J. Genetics **37:** 483–490.
297. Sax, K. & C. P. Swanson. 1941. Am. J. Botany **28:** 52–59.
298. Schmitt, F. O. 1944. Advances in Protein Chem. **1:** 26–68.
299. Schneider, W. C. 1947. Cold Spring Harbor Symp. Quant. Biol. **12:** 169–178.
300. Schomer, H. A. 1936. Biological Effects of Radiation. Ed. by B. M. Duggar. **2:** 1151–1165, McGraw-Hill Book Co., New York and London.
301. Schrek, R. 1946. Radiology **46:** 395–410.
301a. Schrodinger, E. 1945. What Is Life? The Physical Aspect of the Living Cell. Cambridge Univ. Press, Macmillan Co., New York.
302. Schultz, J. 1944. Colloid Chem. **5:** 819–850.
303. Schultz, J. 1947. Cold Spring Harbor Symp. Quant. Biol. **12:** 179–191.
304. Scott, G. H. 1930. Bull. histol. appl. physiol. et path. et tech. microscop. **7:** 251–256.
305. Serra, J. A. 1947a. Portug. Acta Biol., **2:** 45–90.
306. Serra, J. A. 1947b. Cold Spring Harbor Symp. Quant. Biol. **12:** 192–210.
307. Skoog, F. 1935. J. Cellular Comp. Physiol. **7:** 227–270.
308. Smith, G. F. & H. Kersten. 1942. Am. J. Bot. **29:** 785–791.
309. Smith, L. 1942. Am. J. Botany **29:** 189–191.
310. Smith, L. 1943. J. Heredity **34:** 131–134.
311. Smith, L. 1946. J. Agr. Research **73:** 137–158.
312. Smith, L. & R. S. Caldecott. 1948. J. Heredity **39:** 173–176.
313. Sokolov, I. I. 1940. Biokhimiya **5:** 401–407.
314. Sparrow, A. H. 1942a. Can. J. Res. C. **20:** 257–266.
315. Sparrow, A. H. 1942b. Science **96:** 363–364.
316. Sparrow, A. H. 1942c. Proc. Nat. Acad. Sci. **28:** 463–468.
317. Sparrow, A. H. 1944. Proc. Nat. Acad. Sci. U. S. **30:** 147–155.
318. Sparrow, A. H. 1948. Nature **162**(4121): 651–652.
319. Sparrow, A. H., C. L. Huskins, & G. B. Wilson. 1941. Can. J. Research C. **19:** 323–350.
320. Sparrow, A. H. & M. Maldawer. 1950. Proc. Nat. Acad. Sci. U.S. **36:** 636–643.
321. Sparrow, A. H. & F. M. Rosenfeld. 1946. Science **104:** 245–246.
321a. Sparrow, A. H. & R. C. Sparrow. 1949. Stain Techn. **24:** 47–55.
322. Spear, F. G. 1946. Brit. Med. Bull. **4:** 2–10.
323. Spencer, W. P. & C. Stern. 1948. Genetics **33:** 43–74.
324. Spiegelman, S. & M. D. Kamen. 1947. Cold Spring Harbor Symp. Quant. Biol. **12:** 211–223.
325. Spiers, F. W. 1946. Brit. J. Radiol. **19:** 52–63.
326. Stadler, L. J. & F. M. Uber. 1942. Genetics **27:** 84–118.
327. Stein, G. & J. Weiss. 1948. Nature **161:** 650.
328. Stern, K. G. 1947. Yale J. Biol. Med. **19:** 937–949.
329. Stern, H. 1946. Trans. Roy. Soc. Canada Third Series, Sec. V **40:** 141–148.
330. Stern, H. & P. L. Kirk. 1948. J. Gen. Physiol. **31:** 243–248.
331. Strangeways, T. S. P. & F. L. Hopwood. 1926. Proc. Roy. Soc. (London) B **100:** 283–293.
332. Stubbe, H. 1940. Scientia Genetica **1:** 370–384.

333. Sutton, E. 1940. Genetics **25:** 628–635.
334. Svedberg, T. & S. Brohult. 1939. Nature **143:** 938–939.
335. Swanson, C. P. 1942. Genetics **27:** 491–503.
336. Swanson, C. P. 1943. J. Gen. Physiol. **26:** 485–494.
337. Swanson, C. P. 1944. Genetics **29:** 61–68.
338. Swanson, C. P. 1948. Science **107:** 458.
339. Swanson, C. P. & S. H. Goodgal. 1948. Genetics **33:** 127.
340. Swanson, C. P. & A. Hollaender. 1946. Proc. Nat. Acad. Sci. U.S. **32:** 295–302.
341. Swanson, C. P., A. Hollaender, & B. N. Kaufmann. 1948. Genetics **33:** 429–437.
342. Taylor, B., J. P. Greenstein, & A. Hollaender. 1948. Arch. Biochem. **16:** 19–31.
343. Tchaperoff, I. C. C. 1943. Radiology **41:** 61–63.
344. Thoday, J. M. & J. Read. 1947. Nature **160:** 608.
345. Timoffeef-Ressovsky, N. W. & K. G. Zimmer. 1939. Strahlentherapie **66:** 684–711.
346. Timoffeef-Ressovsky, N. W. & K. G. Zimmer. 1947. Biophysic Bd. 1. Das Trefferprinzip in der Biologie. S. Hirzel Verlag. Leipzig.
347. Tytell, A. A. & H. Kersten. 1941. Proc. Soc. Exp. Biol. Med. **48:** 521–525.
348. Vilbrandt, C. F. & H. G. Tennent. 1943. J. Am. Chem. Soc. **65:** 1806–1809.
349. Vintemberger, P. 1928a. Compt. rend. soc. biol. **98:** 536–537.
350. Vintemberger, P. 1928b. Compt. rend. soc. biol. **99:** 1968–1971.
351. von Euler, H. 1946. Svensk. Kem. Tid. **58:** 180–187.
352. von Euler, H. & L. Hahn. 1946. Acta Radiol. **27:** 269–280.
353. von Euler, H. & G. Hevesy. 1942. Kgl. Danske. Videnskab. Selskab. Biol. Med. **17**(8): 1–38.
354. von Euler, H. & G. Hevesy. 1944. Arkiv. Kemi. Mineral. Geol. **17A**(30): 1–60.
355. Waddington, C. H. 1940. Biol. Symp. **1:** 200–214.
356. Wallace, R. H., R. J. Bushnell, & E. H. Newcomer. 1948. Science **107:** 577–578.
357. Warmke, H. E. 1946. Am. J. Botany **33:** 224.
358. Weiss, J. 1944. Nature **153:** 748–750.
359. White, M. J. D. 1935. Proc. Roy. Soc. (London) B **119:** 61–84.
360. Whiting, A. R. 1940. J. Exptl. Zool. **83:** 249–269.
361. Whiting, A. R. 1945a. Biol. Bull. **89:** 61–71.
362. Whiting, A. R. 1945b. Am. Naturalist **79:** 193–227.
363. Whiting, A. R. & C. H. Bostian. 1931. Genetics **16:** 659–680.
364. Wiame, J. M. 1946. Bull. soc. chim. biol. **28:** 552–556.
365. Wichterman, R. 1948. Biol. Bull. **94:** 113–127.
366. Wilbur, K. M. & R. O. Recknagel. 1943. Biol. Bull. **85:** 193–200.
367. Wilson, G. B. & E. R. Boothroyd. 1944. Can. J. Research. C **19:** 400–412.
368. Wilson, W. L. 1946. Biol. Bull. **91:** 229.
369. Woodward, G. E. 1933. Biochem. J. **27:** 1411–1414.
370. Woodward, H. Q. 1932. J. Phys. Chem. **36:** 2543–2553.
371. Wrinch, D. 1940. J. Genetics **40:** 359–377.
372. Wyss, O. W., J. B. Clark, F. Hass, & W. S. Stone. 1948. J. Bact. **56:** 51–57.
373. Zirkle, R. E. 1932. J. Cellular Comp. Physiol. **2:** 251–274.
374. Zirkle, R. E. 1936. Am. J. Roentgenol. Radium Therapy **35:** 230–237.

PROBABLE ULTRASTRUCTURES INVOLVED IN CELL DIVISION*

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One of the basic features of animal cell division is the dependence of the cortex, cleavage furrow, and mitotic apparatus on gelating mechanisms. The gels of the cortex and furrow are optically isotropic. The asters and spindles are also gelated structures, although a portion of the gel, at least, is anisotropic, as indicated by the birefringence of the spindle (Hughes and Swann[1]). This resumé, therefore, is an attempt to resolve the processes of cytokinesis in terms of sol-gel reactions involving mainly the submicroscopic particulates of cytoplasm, which consist of globular proteins, nucleoproteins, and phospholipids (Claude[2]).

The Particulate Nature of Protoplasm. There are several lines of evidence which strongly suggest that the cytoplasmic matrix consists of submicroscopic particulates in addition to those that are microscopically visible. These include: (a) differential vital staining of zones in the matrix fraction of centrifuged *Arbacia* eggs (Harvey[3]); (b) stratification of the cytoplasmic matrix into chromophobic and chromophilic fractions following high speed centrifugation of amphibian and mammalian liver cells (Claude,[4] Brachet[5]); (c) demonstration of submicroscopic spherical particulates in electron micrographs of thin cells grown in tissue culture (Porter and Thompson[6]); (d) isolation of submicroscopic particulates by high speed centrifugal or chromatographic fractionation of cell homogenates (Claude,[2] Riley[7]); and (e) surface chemical properties of cytoplasmic proteins (Kopac[8]).

The Ultrastructure of Protein Gels. Ferry[9] has recently discussed the current views on the nature of protein gels. It is generally agreed that protein gels consist of 3-dimensional networks of protein fibrils. A network of fibrils has been demonstrated in the electron micrographs of fibrin gels by Hawn and Porter.[10]

The major problems involved in protoplasmic sol-gel reactions are not concerned nearly as much with network formation as they are with the formation of fibrils. Protoplasm ordinarily exists as a sol (B in FIGURE 1) and accordingly the presence of extensive fibrillization is contraindicated. The problem, therefore, is to account for the conversion of a solated system into fibrillar and network systems which either spontaneously or by experimental treatment can be readily reverted to the solated state.

Fibrils Formed from Protein Molecules. Wrinch[11] discussed fibrillization of globular proteins as a process involving mainly the linear array of protein molecules which could be joined end-to-end without requiring extensive changes in structure of such molecules. Since then, Waugh[12] produced fibrils and gels from insulin by special procedures. The fibrils could be easily broken down from which reasonably native insulin molecules could

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be recovered; at least, the insulin molecules still retained most of their specific biochemical properties. The end-to-end adlineation of fragments of tobacco mosaic virus obtained by ultrasonic radiation was observed in electron micrographs by Oster.[13] Another important example of fibrillization was described by Szent-Gyorgyi[14] in the reversible formation of fibrous-actin from globular-actin. Obviously, fibrils can be formed reversibly from globular protein molecules.

On the other hand, Monné[15] and Frey-Wyssling[16] have considered that cytoplasmic fibrils are composed of polypeptide bundles. Monné[17] also considered that living fibrils may be regarded as monolayers of parallel oriented polypeptide bundles rolled up like a scroll of paper. This pattern is based on the fibrils manufactured from monolayers of protein molecules by Mazia, Hayashi, and Yudowitch.[18] It is doubtful whether such fibrils would exhibit any degree of reversibility, a requirement if gels are to become

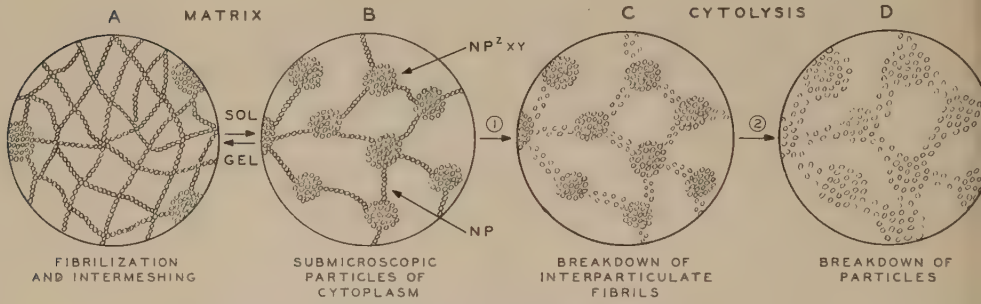

FIGURE 1. Diagrams of probable ultrastructures of cytoplasmic matrix as based on studies of the surface chemical properties of cytoplasmic proteins.[8] (A) Diagram of a network of protein fibrils showing the ultrastructure of a gel. (B) Diagram of cytoplasmic matrix with the consistency of a sol. The spherical submicroscopic particulates, NP^2xy, are mildly restrained by interparticulate protein fibrils, NP. (C) Diagram of the first step in cytolysis, which involves a breakdown of the NP fibrils to low molecular weight proteins. If the media are adequate, [23–28] the cytolytic reactions could be stopped here and thus allow the recovery of the submicroscopic particulates. (D) Diagram of uncontrolled cytolysis, which leads to the breakdown of submicroscopic particulates with the release of low molecular weight proteins. In these diagrams, only protein molecules are shown, although other substances, including nucleic acids and phospholipids, are associated with cytoplasmic proteins and particulates.[2]

sols spontaneously. Furthermore, these fibrils consisting of surface-denatured proteins should be reasonably stable and should therefore be recovered in cell homogenates. Such fibrils have not, as yet, been found in carefully prepared homogenates.

Probable Mechanisms of Intracellular Fibrillization. On the basis of surface chemical studies on the properties of cytoplasmic proteins, Kopac[8] has postulated that the cytoplasmic matrix consists essentially of a suspension of submicroscopic particulates—the more or less symmetrical aggregates of proteins and other molecules (B in FIGURE 1). This kind of architecture would provide the consistency of a sol. The fibrillization of proteins within these aggregates and subsequent network formation would lead to gelation (A in FIGURE 1). Solation would occur by the reversion of the linear aggregates to spherical aggregates. The general scheme of the sol-gel reaction would be:

Spherical Aggregates

Fibrillization ↓↑ *Defibrillization*

Linear Aggregates

Network formation ↓↑ *Breakdown of network*

3-Dimensional Network

The formation of linear aggregates (fibrils) from the spherical aggregates could arise in one of two ways, depending on the forces which hold the aggregate together. On one hand, an aggregate could be held together by weak lateral forces (A in FIGURE 2). In these aggregates, fibrillization would be complex, *e.g.*, requiring first the formation of end-to-end bonds between the globular protein molecules, the disruption of weak lateral forces, and subsequent linear unravelling to form a fibril.

On the other hand, the molecules within the aggregate could be held together by strong end-to-end forces, in addition to weak lateral forces (B in FIGURE 2). In these aggregates, the fibrils are preformed. Thus, fibrillization would require the release of the weak lateral forces and the subsequent unravelling of the preformed fibrils. In either situation, gelation would follow the formation of 3-dimensional networks from the fibrils (A in FIGURE 1).

Solation would require the disruption of the network and defibrillization. The fibril could disappear by coiling up with the re-establishment of lateral forces, thus becoming the preformed fibril with the gross architecture of a spherical, submicroscopic particulate (B in FIGURE 2). On the other hand, the end-to-end forces could be dissipated, with consequent collapse of the fibril. A new spherical aggregate could be formed, provided that the necessary lateral directing forces were set-up between the molecules released by breakdown of the fibril.

As shown in FIGURE 1, C and D, and in FIGURE 2, C, disruption of both lateral and end-to-end forces would cause the breakdown not only of a gel but of the aggregates as well. This effect would follow cytolysis or the intentional disintegration of a cell in a nonphysiological medium. In the latter state, the proteins would be clearly susceptible to surface denaturation.[8]

Significance of the Cleavage Substance. The most striking evidence in support of the existence of a cleavage substance is shown by the delay in cleavage of fertilized clear quarters of *Arbacia* eggs (Harvey[19]), of centrifuged *Dendraster* eggs (Moore[20]), and of ultracentrifuged *Ascaris* eggs (Beams[21]). A similar delay in cleavage can be induced by application of medium hydrostatic pressures (Marsland[22]).

If one assumes that the cleavage substance is the denser cytoplasmic particulate fraction, capable of fibrillization at the cortex and furrow (*i.e.*, spherical submicroscopic particulates with preformed fibrils), then it should be possible to induce a deficiency of such gel-forming substances in the centripetal regions of a cell by prolonged centrifugation.

The delay or inhibition of cleavage furrow formation, as produced by application of hydrostatic pressures,[22] could involve a suppression of fibrillization of the preformed fibrils. On the other hand, the action of pressure need not necessarily be direct. It is possible that pressure may modify the mechanisms responsible for the energy required to form fibrils or to maintain fibrils and networks.

Energy could conceivably be utilized not only in forming end-to-end bonds but also in disrupting the weak lateral forces which maintain the spherical aggregates. Obviously, the spherical aggregates offer greater ran-

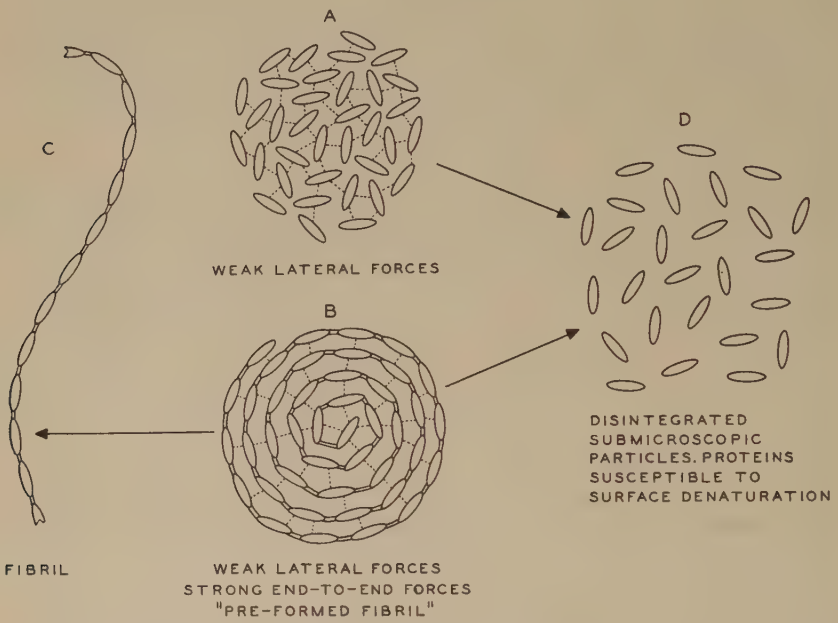

FIGURE 2. Diagrams showing relationship between submicroscopic particulates, A and B, and a fibril, C. Submicroscopic particulate, A, consists of proteins held together by weak lateral forces. Submicroscopic particulate, B, in addition to the weak lateral forces, possesses strong end-to-end forces between the protein molecules. This comprises a preformed fibril. Fibrillization involves the breakdown of weak lateral forces and subsequent unravelling to form the fibril, C. Defibrillization requires the coiling up of the fibril to reform the spherical aggregate, B. Loss of lateral and of end-to-end forces would lead to disintegration either of the particulates, A and B, or of fibril, C, thus releasing low molecular weight proteins as in D. In the diagrams, only protein molecules are included.

domness to their components than the fibrils. Moreover, the aggregate with only weak lateral forces between its components has greater randomness than one with a preformed fibril. Unless these configurations have an unusual kind of entropy, maintenance of the fibrils and of the network would require energy because of restricted randomness. In this connection, the entropy of chain configurations has been discussed by Ingersoll and Johnson[23] and by Hermans.[24]

At present, there is no known mechanism to explain the nature of energizing either cytoplasmic fibrillization or gelation. As a first approximation, one may assume that adenosine triphosphate (ATP) is involved. For

example, Munch-Petersen[25] reported that ATP expands monolayers of myosin. The action of ATP probably facilitates the unfolding of the myosin molecule and thus enhances the spreading of the myosin monolayer. One might postulate that a pre-fibril could be formed if enough of a protein molecule were unfolded at its terminal positions, for example, by the action of ATP, to permit subsequent end-to-end bonding with other similar units, *e.g.*, by van der Waals forces interacting between the exposed nonpolar amino acid residues. The role of ATP in fibrillization and gelation has not as yet been directly established, however, and it may not be for some time.

The Basic Problems. One of the major problems currently confronting biochemists and cell physiologists is the development of media into which cells can be disintegrated without producing drastic changes in the microscopic and submicroscopic cytoplasmic particulates. Our approach to the problem is micrurgical. For example, test media are injected into the cytoplasm of various cells and the effects on sol-gel reactions and on the visible condition of cytoplasmic inclusions are studied. This has led to the development of an intracytoplasmic medium consisting of KCl 0.042M + NaCl 0.017M + $CaCl_2$ 0.0015M + $MnCl_2$ 0.001M, which is reasonably satisfactory in maintaining the integrity of cytoplasmic particulates on injection into amoebae (Kassel and Kopac[26]). This medium, however, does not prevent solation of the plasmagel of *Pelomyxa* (Kopac[27]). Another approach is to remove nuclei from the amoeba by micrurgical means and to study the cytological changes that may appear when these nuclei are transferred to test media (Kassel and Kopac[28]).

One of the probable harvests, therefore, if proper intracytoplasmic media can be developed, would be the isolation of submicroscopic particulates (spherical aggregates). Not only might these aggregates retain their specialized biochemical properties, *i.e.*, enzymes and enzyme teams, energy-synthetic coupling mechanisms, *etc.*, it also might be possible to induce some of them to undergo reversible fibrillization. Actually, the attempts here would be to isolate the so-called cleavage substance. If the latter can be achieved, then here might be a direct approach towards clarifying the energetics of fibrillization and gelation, as well as the mechanisms of cytokinesis.

Bibliography

1. Hughes, A. F. & M. M. Swann 1948. J. Exptl. Biol. **25:** 45.
2. Claude, A. 1946. J. Exptl. Med. **84:** 51.
3. Harvey, E. B. 1941. Biol. Bull. **81:** 114.
4. Claude, A. 1943. Biol. Symp. **10:** 111.
5. Brachet, J. 1950. Ann. N. Y. Acad. Sci. **50:** 861.
6. Porter, K. R. & H. P. Thompson. 1947. Cancer Research **7:** 431.
7. Riley, V. T. 1948. Science **107:** 573.
8. Kopac, M. J. 1950. Ann. N. Y. Acad. Sci. **50:** 870.
9. Ferry, J. D. 1948. Advances in Prot. Chem. **4:** 1.
10. Hawn, C. van Z. & K. R. Porter. 1947. J. Exptl. Med. **86:** 285.
11. Wrinch, D. 1940. J. Genetics **40:** 359; 1941. Cold Spring Harbor Symposia Quant. Biol. **9:** 218.
12. Waugh, D. F. 1941. Proc. Amer. Physiol. Soc. **133:** 484; 1948. Anat. Rec. **101:** 656; 1948. J. Amer. Chem. Soc. **70:** 1850.

13. Oster, G. 1947. J. Gen. Physiol. **31:** 89.
14. Szent-Gyorgyi, A. 1948. Nature of Life. 91 pp. Academic Press. New York.
15. Monne, L. 1948. Advances in Enzymology **8:** 1.
16. Frey-Wyssling, A. 1948. Submicroscopic Morphology of Protoplasm and Its Derivatives. 263 pp. Elsevier. New York.
17. Monne, L. 1949. Arkiv Zool. [A] **42:** 1.
18. Mazia, D., T. Hayashi, & K. Yudowitch. 1947. Cold Spring Harbor Symposia Quant. Biol. **12:** 122.
19. Harvey, E. B. 1946. J. Exptl. Zool. **102:** 253.
20. Moore, A. R. 1938. Proc. Soc. Exptl. Biol. Med. **38:** 162.
21. Beams, H. W. 1951. Ann. N. Y. Acad. Sci. **51**(8): 1349.
22. Marsland, D. 1938. J. Cell. Comp. Physiol. **12:** 57.
23. Ingersoll, H. G. & A. A. Johnson. 1948. Nature **162:** 225.
24. Hermans, J. J. 1947. Trans. Faraday Soc. **43:** 91.
25. Munch-Petersen, A. 1948. Nature **162:** 537.
26. Kassel, R. & M. J. Kopac. 1949. Anat. Rec. **105:** 106.
27. Kopac, M. J. 1950. Cancer Research **10:** 229.
28. Kassel, R. & M. J. Kopac. 1950. Anat. Rec. **108:** 85.

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